On Roles of Cholinergic Amacrine and Müller Glial Cells in the Development of Networks in the Inner Plexiform Layer of the Chick Retina

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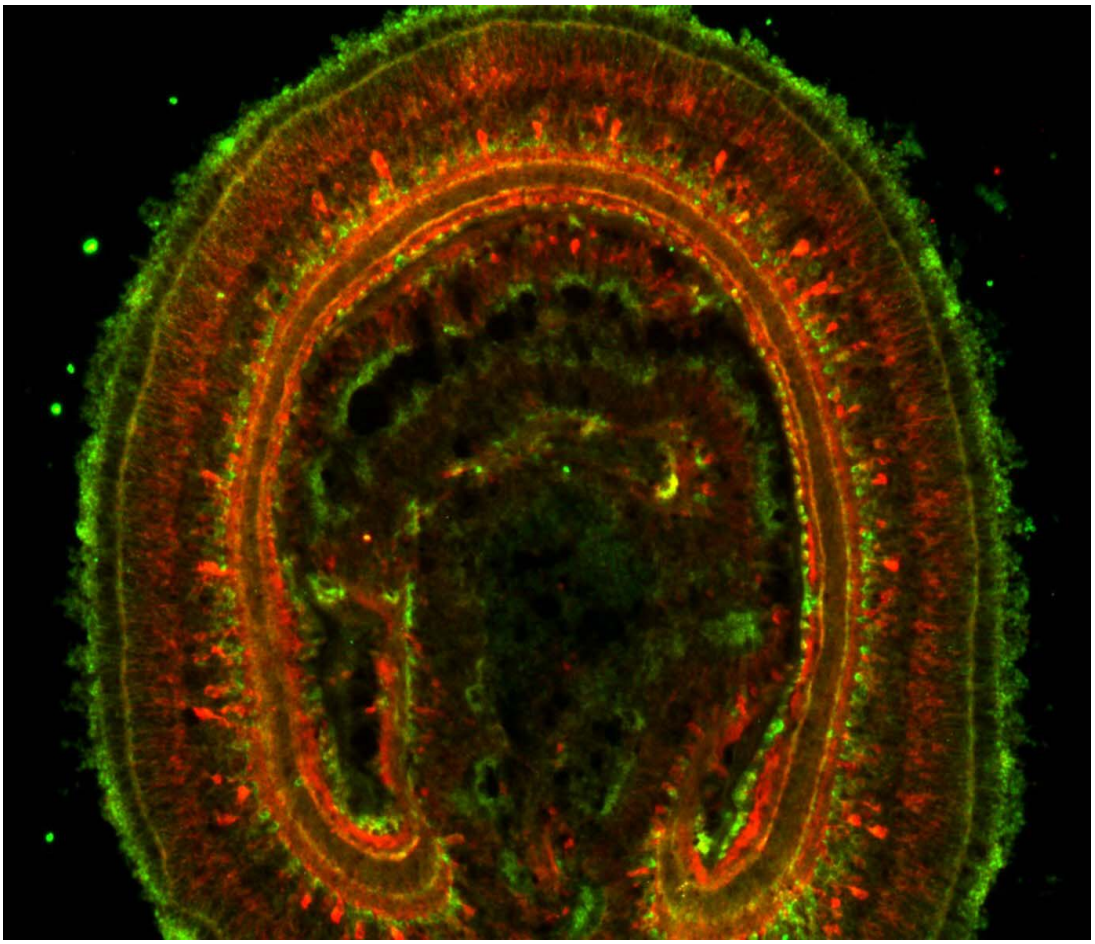
This thesis is dedicated to my parents



Known is a drop, unknown is an ocean……..

Picture information: A retinal explant immunostained for ChAT (green) and CRABP (red).

Summary

The inner plexiform layer (IPL) of the Vertebrate retina is a highly organized synaptic region amassed with a myriad of processes from different cell types, like ganglion cells (GCs), amacrine cells (ACs) and bipolar cells (BCs). Their dendrites stratify at different levels within this cell-free zone, e.g. so-called subbands. Further, Müller glial cells (MCs) span radially through all retinal layers. In this study, I have focused on cholinergic ACs and MCs, and their possible roles in the formation of the IPL in the embryonic chicken retina.

In the first chapter I have analysed the *in vivo* development and differentiation of starburst amacrine cells (SACs) and their role in cholinergic stratification. Type-I and type-II SACs are mirror-symmetrically arranged ACs on both sides of the IPL, which send their processes into the latter, where they stratify as subbands ‘a’ and ‘d’. Acetylcholine is the predominant neurotransmitter of these cells, e.g. they are cholinergic. Using the cholinergic markers choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), I show here immunohistochemically that an early postmitotic pool of cells in the inner half retina expresses first AChE, and then also ChAT. SACs and GCs are derived from this pool. By embryonic stage E6, two narrow rows of $ChAT^+$ cells appeared in the future IPL, separated by a narrow band of AChE. Thus, AChE might be playing a guiding role for $ChAT^+$ processes. Type-I SACs retained their high $ChAT^+$, but not AChE expression, which was less pronounced in type-II SACs. Remarkably, type-I SACs in the INL were neighbored by strongly AChE-reactive cells which were $ChAT^-$, a finding whose functional implications needs further attention.

In chapter II, I show that the earliest $ChAT^+$ cells also co-expressed the ganglion cell-specific marker Brn3a and CRABP. By E6, one set of cells down-regulated both ChAT and CRABP, but up-regulated Brn3a, marking the specification of GCs, while another set of cells reacted to the contrary, marking the specification of the two future types of SACs. By E8, type-I cells at the IPL/INL border lost their expression of CRABP, while type-II SACs in the IPL retained this protein. A co-localization of the early GC marker Brn3a with $ChAT^+$ cells revealed that early GCs transiently express ChAT. Throughout these developmental stages, both the GCs and the ACs expressed AChE.

Now following, I have performed *in vitro* experiments to analyze the role of SACs and Müller glial cells during IPL development, using both 3D retinal spheroids and retinal explants. In chapter III, I describe the establishment of a novel explant culture system, presenting the development of all retinal layers of an in vivo retina, except the GCL. Although being used here for studies of the inner retina, it is worthwhile to mention that with these explants the initial formation of photoreceptor outer segments can be followed.

In continuation to the above mentioned in vivo studies, in chapter IV, I have similarly analyzed IPL formation by using both in vitro systems. Since type-II SACs are closely associated with GCs, I postulated that GCs could influence their differentiation, and in turn the development of the IPL as well. To test this hypothesis, I investigated the development of the IPL in explants, in which GCs are completely lost due to the absence of an optic nerve. In addition, 3D retinospheroids were used, where cells are completely dissociated and allowed to form histotypical structures, but again mostly in absence of GCs. Interestingly, in both *in vitro* culture models the IPL still developed, including both cholinergic subbands and migration of type-II SACs. This is strong evidence that major parts of the IPL develop in absence of GCs.

Besides SACs, early Müller glial cells (MCs) have been suggested to provide guiding cues to establish the neuritic plexus of the IPL. Therefore, in the last chapter V, I have used the specific gliotoxin DL-alpha aminoadipate (AAA) in retinal spheroids to target MCs and thereby analyze the role of MCs during IPL development. At a sub-toxic concentration of 0.4 mM AAA, though the IPL was developed, the cholinergic stratification was severely disturbed, indeed suggesting an organizing role of MCs during IPL development. Moreover, thereby the numbers of inner retinal cells increased, while photoreceptor differentiation was inhibited, indicating further roles of MCs in cell lineage regulation.

Zusammenfassung

Die innere plexiforme Schicht (IPL) der Wirbeltierretina ist eine hoch organisierte synaptische Zone, welche sich aus unzähligen Fortsätzen verschiedener Zelltypen, wie den Ganglienzellen (GCs), Amakrinzellen (ACs) und Bipolarzellen (BCs), zusammensetzt. Deren Dendriten sind in dieser zellfreien Zone geschichtet als so genannte Subbanden angeordnet. Ferner durchqueren Müllerzellen (MCs) die ganze Retina, also auch die IPL, in radialer Richtung. Ich habe mich in dieser Studie mit cholinergen ACs sowie mit MCs, und deren möglichen Funktionen bei der Bildung der IPL in der embryonalen Hühnchen-Retina beschäftigt.

In Kapitel I habe ich die Normalentwicklung und Differenzierung von *Starburst Amacrine Cells* (SACs) und deren Rolle bei der cholinergen Stratifizierung analysiert. Typ-I und Typ-II SACs sind spiegelsymmetrisch auf beiden Seiten der IPL angeordnete ACs, welche ihre Fortsätze in die IPL senden, wo sie jeweils in die Subbanden ´a´ bzw. ´d´ projizieren. Sie benützen Acetylcholin als Neurotransmitter, sind also cholinerg. Mit Cholinacetyltransferase (ChAT) und Acetylcholinesterase (AChE) als cholinerge Marker habe ich immunhistochemisch gezeigt, daß eine frühe Population postmitotischer Zellen der inneren Retina zunächst AChE, und dann ChAT exprimiert; von ihr stammen GCs und SACs ab. Um das Embryonalstadium E6 erscheinen zwei Reihen von $ChAT^+$ Zellen in der zukünftigen IPL, die von einer engen $AChE^+$ Bande getrennt sind. AChE könnte also eine Leitfunktion für ChAT-positive Fortsätze ausüben. Die Typ-I SACs behielten ihre ChAT-, aber nicht ihre AChE-Expression, bei. Dies war bei Typ-II SACs weniger ausgeprägt. Auffällig war eine enge Nachbarschaft von Typ-I SACs mit stark AChE-exprimierenden Zellen in der INL, deren Funktion noch weiter untersucht werden muß.

In Kapitel II habe ich gezeigt, daß die frühesten $ChAT^+$ Zellen auch den GC-Marker Brn3a und CRABP koexprimieren. Um E6 regelte ein Teil dieser Zellen ChAT und CRABP herunter und Brn3a gleichzeitig herauf, was die Differenzierung der GCs anzeigt, während ein anderer Teil gegenteilig reagierte, was auf die Spezifizierung der zukünftigen SACs hinweist. Um E8 haben die Typ-I-Zellen an der INL/IPL-Grenze ihre CRABP-Expression verloren, während Typ-II SACs dieses Protein beibehielten. Die Kolokalisierung von Brn3a und ChAT hat ge-

zeigt, daß frühe GCs ChAT transient exprimieren. AChE wurde während dieser Stadien sowohl von GCs wie auch von ACs exprimiert.

Nun folgend habe ich Experimente durchgeführt, um die Rolle von SACs und MCs bei der IPL-Entwicklung zu analysieren, wobei sowohl retinale Sphäroide und Explantate eingesetzt wurden. In Kapitel III habe ich die Entwicklung eines neuartigen Explantsystems beschrieben, welches die Entwicklung aller Retinaschichten, außer der Ganglienzellschicht, in vitro darstellt. Obwohl dieses System hier zum Studium der inneren Retina eingesetzt wurde, sollte erwähnt werden, daß mit diesen Explantaten auch die Bildung der Außensegmente von Photorezeptoren verfolgt werden kann.

In Kapitel IV habe ich die obigen in vivo-Studien hinsichtlich der IPL-Bildung entsprechend an beiden in-vitro-Systemen fortgeführt. Da Typ-II SACs eng mit GCs assoziiert sind, habe ich postuliert, daß GCs deren Differenzierung, und somit auch die der IPL beeinflussen könnten. Daher habe ich die Entwicklung der IPL in Explantaten untersucht, in denen die GCs als Folge der Abtrennung des optischen Nervs vollständig absterben. Dasselbe wurde mit retinalen Sphäroiden durchgeführt, bei denen histotypische Strukturen aus vollständig dissoziierten Zellen entstehen, aber wiederum praktisch keine GCs enthalten. Interessanterweise entwickelte sich in beiden in vitro-Systemen eine IPL, inklusive der beiden Subbanden und wandernden Typ-II SACs. Dies macht deutlich, daß sich wesentliche Teile der IPL in Abwesenheit von GCs entwickeln können.

Neben SACs wird vermutet, daß auch MCs Leitstrukturen darstellen könnten, um den Neuritenplexus der IPL zu etablieren. Daher habe ich in Kapitel V das spezifische Gliotoxin DL-Alpha-Aminoadipate (AAA) in retinalen Sphäroiden eingesetzt, um damit die Rolle der MCs bei der IPL-Bildung zu analysieren. Bei einer subtoxischen Konzentration von 0,4 mM AAA hat sich die IPL zwar gebildet, jedoch war die cholinerge Stratifizierung stark gestört, was tatsächlich eine organisierende Rolle der MCs während der IPL-Entwicklung nahelegt. Ferner hat sich dabei die Anzahl der inneren Retinazellen erhöht, während die Zahl der Photorezeptoren erniedrigt wurde, was weitere Funktionen der MCs bei der Regulation der Zellgenealogie nahelegt.

General Introduction

The eye is the central organ that is associated with vision and its main function is to focus the light from the external sources and transmit it to the brain in order to accomplish the sense of vision. Given its biological importance to a wide variety of animals, human beings possess an extraordinary ability to perceive color and shape, and also to differentiate between light intensities (Sung and Chuang, 2010). For the accomplishment of vision, the light must fall on the retina, which is a 0.2 mm thick central nervous tissue where the perceived light energy is converted to a chemical energy by light sensitive cells, the photoreceptors.

Development of the retina

The retina is a part of the central nervous system which is derived from the neural tube and it is an ideal region of the vertebrate brain to study the structure and function of CNS. The retina is formed during development of the embryo from the optic vesicles when they out-pouch from two sides of the developing neural tube. The early optic vesicles fold back in upon themselves to form the optic cup with the inside of the cup becoming the retina and the outside becoming the retinal pigment epithelium (RPE). Initially both walls of the optic cup are one cell thick, but the cells of the inner wall divide several times to form a stratified neuroepithelial layer that later becomes the retina.

Structure of the retina

The vertebrate retina consists of five types of neuronal cells, namely the ganglion cells, amacrine cells, bipolar cells, horizontal cells and the outer most photoreceptor cells which comprise the rods and cones. Apart from these cells, it also contains a special type of glial cells, called the Müller cells, which are found only in retina. Each of the cell type is connected with the other cell types through synapses which are involved in neurotransmission. The cells are arranged in discrete layers called the nuclear layers. The photoreceptors occupy the outer portion of the retina and form the outer nuclear layer (ONL). The horizontal, bipolar and amacrine cells together comprise the inner nuclear layer (INL) and the ganglion cells occupy the inner most ganglion cell layer (GCL).

The synapses of the different cells are organized into distinct laminas. In the outer retina, photoreceptors contact horizontal cells and bipolar cells within a single lamina and is called the outer plexiform layer (OPL) which separates the ONL and the INL. Within the inner retina, synapses between the bipolar and amacrine interneurons and their post synaptic partners, the retinal ganglion cells are localized to the inner plexiform layer (IPL).

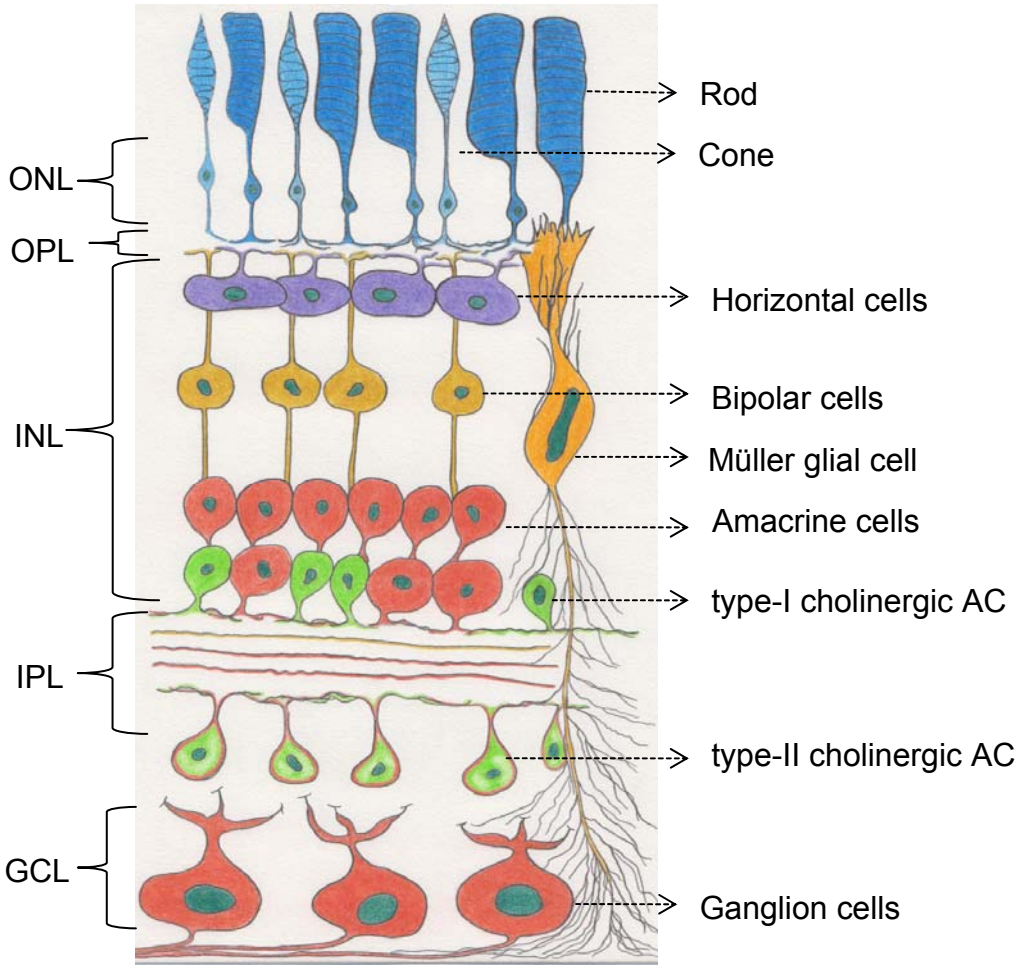


Fig. 1: Schematic representation of the structure of retina. Two special types of amacrine cells, type I and type II cholinergic starburst amacrine cells are shown (green cells) which play major roles in the development of an IPL.

The Müller cells spread throughout, spanning the entire retina. The IPL is wider than the OPL and is a highly ordered structure. As mentioned above, all the synapses are arranged in discrete levels called the sublayers or sublaminas which play a vital role in the neurotransmission. Because of its high order of organization, the IPL provides an immense opportunity to understand the developmental processes of such complex structures.

In this dissertation, I have mainly focused on the development of the cholinergic system with respect to the two important members of the cholinergic components ChAT and AChE which are involved in the synthesizing and degradation of the neurotransmitter acetylcholine, respectively, and especially the role of AChE in development of an IPL in chick retina. All the neurotransmitter systems in retina have been vastly studied but the cholinergic system has always been fascinating for developmental biologists mainly due to the enzyme AChE which received enormous importance for its non-enzymatic and non-classical functions during early embryonic development.

Cholinergic neurotransmission

Chick retina like other vertebrates contains glutamate, GABA, glycine, serotonin, dopamine, acetylcholine etc. as neurotransmitters. GABA and glycine are the classical inhibitory neurotransmitters (Marc, et al., 1995). Though all the neurotransmitters have their own importance in the retina, acetylcholine has been studied extensively since long. It is not a major neurotransmitter in the vertebrate retina but still considered as a classical fast excitatory neurotransmitter of the peripheral nervous system. A simplified model of a classical cholinergic neurotransmission is shown below.

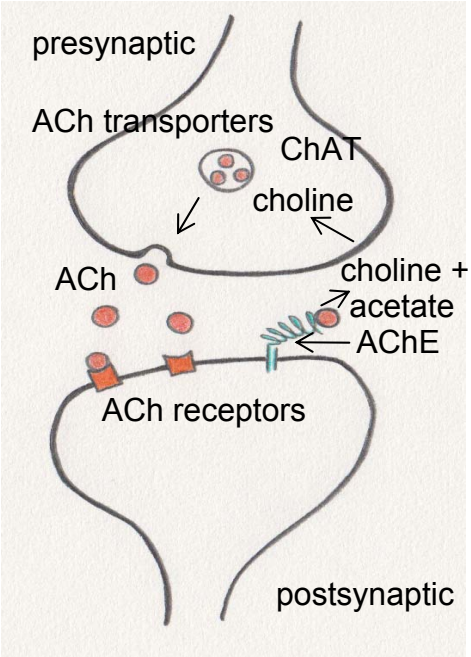


Fig. 2: Cholinergic neurotransmission. A simple model of a cholinergic neurotransmission carried out at the synapse and the components involved in the neurotransmission.

The cholinergic neurotransmission is a well established neurotransmitter system that has been studied since the beginning of the last century. The cholinergic system consists of the neurotransmitter acetylcholine (ACh), choline acetyltransferase (ChAT), the enzyme which is required for the sysnthesis of ACh, the receptors for ACh which are two types, namely the muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) which are involved in the breakdown of ACh. The cholinergic system could be considered completely established when the transmitter itself (Lindeman, 1947), its synthesizing enzyme choline acetyltransferase (ChAT) (Hebb, 1955), its degradative enzyme acetylcholineaterase (AChE) (Chen et al., 1956), high acetylcholine uptake and its release functions have been developed (Neal and Gilroy, 1975).

The cholinergic system in IPL development

Though the classical function of the cholinergic system is to take part in the neurotransmission, most of the cholinergic components are found to be expressed much earlier even before synaptogenesis takes place. In human, the nAChRs, ChAT and ACh appear much earlier than the muscarinic receptors appear (Gremo et al., 1987; Ravikumar and Sastry, 1985). As the main function of AChE is to hydrolyze ACh, in many species, both vertebrate and invertebrate, the enzyme is found to be expressed much earlier even when ACh is not yet produced (Karczmar, 2007). In chick, AChE appears very early in neurogenesis, before the onset of transmission, function or synaptogenesis both in brain and retina (Shen et al., 1956; Layer, 1983, 1990, 1991; Layer and Willbold, 1995). In the chick ACh and ChAT were observed in the neuroblasts and neural crest at 2.5 days of development (Greenberg and Schrier, 1977). This is also true for the CNS outgrowth, the retina. AChE appears in the inner plexiform layer long before synaptogenesis during chick retinal development (Shen et al., 1956).

In embryonic chick retina, the IPL is the first area that begins to stratify and the process begins at around E8. AChE is expressed around E3/E4 in the early vitreal cells and the expression is high in the early IPL-like area before the synapses could be localized. In the IPL, AChE is localized in at least five different sublayers out of which two are involved in cholinergic transmission which has

been exclusively studied using AChE histochemistry by Layer et al. during 1980s and 90s. They have also clearly suggested the importance of AChE in the formation of ON and OFF channel dichotomy which is related to retinal physiology. Different sets of bipolar, amacrine and ganglion cells transmit responses to either the onset of light (serving the ON channel) or cessation of light (serving the OFF channel). Extensive reports are already available on the formation of these ON and OFF stratification in different species (Famiglietti and Kolb, 1976; Nelson et al., 1978; Günhan et al., 2000; Chalupa and Günhan, 2004). The IPL with its wide cell-free zone and multi-layered stratification provides an excellent example of complex but highly organized structures of neurons and their processes.

In the vertebrate retina, acetylcholine (ACh) is found in the mirror symmetric pairs of amacrine cells described above. Acetylcholine (ACh) is not a predominant neurotransmitter in the vertebrate retina. However, ACh appears to play not only a decisive role in retinal physiology, but already so during network formation of the inner retina (Baughman and Bader, 1977; Layer, 1983; Marc, 1986; Hutchins, 1987). One of the mirror pairs occurs in the amacrine cell layer with dendrites in sublamina **a** (OFF sublamina of the IPL). The other of the pair has its cell body displaced to the ganglion cell layer and its dendrites stratify in sublamina **d** (ON sublamina of the IPL).

The IPL – a special zone of stratified layers

The multi-stratified area of the IPL is a highly organized network of interconnecting synapses from different types of retinal neurons. It has been of great interest for the developmental biologists to understand the formation of such a high order of organization that would provide a better opportunity to understand the development of the brain. Recent advancements in immunohistochemical techniques have helped much better to reveal the development of these synaptic layers by employing suitable antibodies against specific proteins expressed in the zone. The axonal endings of bipolar cells transmit information which is received from the photoreceptors to the neuropil of the inner plexiform layer (IPL) where they communicate with different varieties of functionally specialized amacrine cells and to dendrites of the various ganglion cells (Kolb et al., 1992).

Cells involved in the development of the IPL

There are many different types of amacrine and ganglion cell branching in the IPL of the vertebrate retina. From early Golgi-staining studies, at least 25 to 60 different amacrine cell types have been reported in the monkey and human retinas (Mariani, 1990; Kolb et al., 1992; 1995). Though it is not yet clearly known whether all these different types of amacrine and ganglion cells stratify the IPL, a special type of amacrine cells called the starburst amacrine cells which are the only cholinergic amacrine cells in the retina are found to widely stratify the IPL even at early developmental stages (Famiglietti, 1983; Masland, 1988).

Starburst amacrine cells

In the rabbit retina, pairs of specialized amacrine cells arranged mirror symmetrically were identified on both the side of IPL and such cells have been named starburst cells (Famiglietti, 1983; Masland and Tauchi, 1986; Prada et al., 1999). Starburst ACs (SACs) are cholinergic cells and are the first to establish separate ON and OFF sublaminae within the IPL, and can be traced by ChAT (choline acetyltransferase), the enzyme needed for the synthesis of the neurotransmitter acetylcholine. There is strong evidence that these cholinergic SACs are most important for the entire network formation of the vertebrate retina. In the adult chicken retina, a third type of cholinergic cell was reported which developed relatively late when compared to the other two cell types (Millar et al., 1985, 1987). These starburst amacrine cells are mainly involved in the cholinergic neurotransmission in the retina.

Müller glial cells and their role in IPL development

Müller cells are the only principal glial cells of the retina in birds. They form architectural support structures stretching radially across the thickness of the retina and form the outer and inner limiting membrane, respectively. The cell bodies of Müller cells sit in the inner nuclear layer and project irregularly thick and thin processes in either direction to the outer limiting membrane and to the inner limiting membrane. Müller cell processes insinuate themselves between cell bodies of the neurons in the nuclear layers and envelope groups of neural processes in the plexiform layers. Both the Müller glial and retinal neuronal cells

are produced from a single progenitor cell (Turner and Cepko, 1987). The generation of neurons occurs in two phases, the first involves the production of ganglion, cones, amacrine and horizontal cells while the second phase involves the production of bipolar, rod photoreceptors and Müller glial cells (Reichenbach and Robinson, 1995). The generation of cells in both the phases occurs in the apical side, and the cells need to migrate to their corresponding positions. Though Müller cells differentiate very late during embryonic development, their early processes and trunks are thought to guide much of the neuron migrations and direct the neurite differentiation.

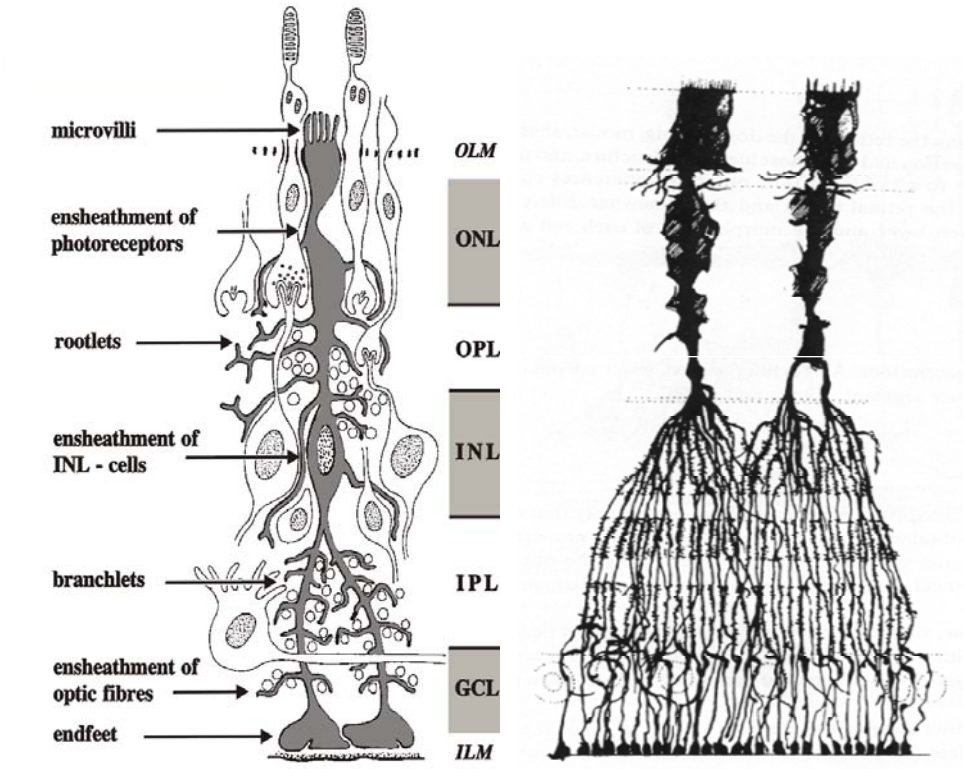


Fig. 3: Schematic representation of the relation between retinal neurons and Müller glial cells. Note the Müller cell processes extending from the inner limiting membrane to the outer limiting membrane. All the neuronal cell types are surrounded by the glial cells. On the right, fine processses of Müller glial processes observed by the Golgi method are shown (Cajal, 1892). For abbreviations, see List of Abbreviations.

Apart from their physical presence, the role of Müller glial cells has really astonished the developmental biologists since a couple of decades. A crucial requirement for a possible developmental function of Müller cells is expression of molecules which enable them to efficiently communicate with other cells. It has

already been shown that the Müller cells express receptors in order to respond to a wide range of autocrine and paracrine signals (Hicks et al., 1992; reviewed by Willbold and Layer, 1998). They possess receptors to various growth factors such as nerve growth factors (NGF; Chakrabarti et al., 1990), epidermal growth factors (EGF), transforming growth factors (TGF; Sagar et al., 1991), etc. Müller cells also play important roles during the final maturation of photoreceptors, as was shown by using a specific glia toxin, DL-alpha aminoadipate (Rich et al., 1995). The branchlets of the long Müller glial processes in the IPL could also support the stratifications in the IPL. In vitro, high concentration of DL-alpha aminoadipate induced total damage of Müller cells leading to a complete breakdown of histogenesis (Olney et al., 1971; Kato et al., 1990; Reinicke and Layer 1992; Germer et al., 1997).

Since Müller glial cells offer such structural scaffolds even at earlier stages, they might have important roles in the development of the IPL and its stratification as well.

The Chick – A great model system

Embryonic development is a tremendously complex process, which has fascinated man since the beginning of developmental biology studies. How does fertilization result in the formation of a complete, independent individual? Where is the information for this complexity encoded, and what mechanisms ensure that it is decoded appropriately at times necessary? To answer these fundamental questions, science has made use of a number of “model systems,” each with different advantages in that they allow various experimental approaches to different extents. The chicken egg is such a common and accessible source of embryo and it has been quoted by Wolpert as “the source of embryo that attracted the interest of ancient Egyptians as well as of Aristotle, who opened eggs at different stages of incubation to examine the progression of development” (Wolpert, 2004).

Analysis of the retinal development through various stages of embryonic growth in chick gives a better understanding of neurobiology. As one of the most simply structured parts of the central nervous system and also because it is located outside the skull which makes it easily accessible, the retina is often used in experiments. Since studies on humans, however, are generally not possible or

practical, other animals are frequently used. The chick embryo is often a prime choice because it is a higher vertebrate that can be easily manipulated and observed during the entire process of embryonic growth through the use of eggs or cultures (Stern, 2005).

Retinospheres – 3D culture systems

"Self-organization is certainly one of the stunning basic features of living organisms at all scales of organization, from molecular to population level. This becomes particularly evident with developing of regenerative cell systems" (Layer et al., 2010). The self-organizing capacity of certain cells to reassemble and reform tissues or even whole animals refers historically to the introduction of the so-called reaggregation approach, which nowadays represents the basis of all tissue engineering. Such organotypic cultures present the major advantage over conventional monolayer cultures, in that their growth of cells develops into histotypic 3-dimensional tissues (Layer and Willbold, 1994; Layer et al., 2002). Organotypic cultures have been widely used to develop engineered tissues that eventually can be employed in clinical therapy; typically, they are based on reaggregated spheres.

Reaggregated cell spheres can form functional tissues

With the reaggregate approach it is attempted to achieve – under controlled culture conditions – a complete reconstruction of tissues from dispersed cells of a particular origin. Technically, a tissue of interest is enzymatically or mechanically dissociated to single cells. The dispersed cells are reaggregated under proper culture conditions and by constant rotation into cellular spheres.

The reconstruction of retina in vitro

In order to advance various applications for tissue engineering, it is essential to understand how a tissue can be constructed step by step from completely dispersed cells in vitro. The vertebrate retina represents the most powerful reconstruction model developed to date for the analysis of tissue regeneration and also for other pharmaceutical and toxilogical studies. Fully dissociated cells of the embryonic avian retina have the capacity to reconstitute different types of spheres (retinospheroids and stratospheroids) with a more or less complete ar-

rangement of retinal layers, allowing the analysis of basic principles of neural layer formation (Layer et al., 2010).

Retinal explants as developmental model systems

After retinospheres which served as model systems since years, novel explants culture systems were introduced during the period of this study which almost mimics the in vivo system (Thangaraj et al., 2011). The main advantage of this system is that it has been developed without any supporting materials and the cultures could be maintained for longer periods in vitro allowing us to follow the developmental stages like that of in vivo. E6 embryonic chicken retinas were used to produce explants. The details of the methods of producing the explants and its advantages are explained in chapter III.

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Aims of the study

The main aims of this thesis were based on the following questions:

1. In vertebrate retina the starburst amacrine cells are the only cholinergic cells that produce acetylcholine and they contribute significantly to the physiological functions of the retina. When and how do these early amacrine cells become cholinergic in the chick embryo retina?
2. The ganglion cells are the first to differentiate in any vertebrate retina and are closely followed by starburst amacrine cells. Do ganglion and starburst cholinergic amacrine cells differentiate from a common pool of progenitors or do they arise from separate cell populations?
3. AChE is a multi-faceted molecule which is expressed very early in the retina even before ACh is synthesized. Does early expression of AChE play a significant role in the differentiation of the cholinergic amacrine cells and development of the inner plexiform layer (IPL)?
4. As the ganglion cells differentiate they migrate to the GCL which is followed by type-II cholinergic amacrine cells in quick succession leading to the formation of the IPL. Is development of the IPL dependent on ganglion cells?
5. In vitro, ganglion cells can be knocked out by optic nerve transection. Can the cholinergic amacrine cells differentiate and stratify the IPL in absence of ganglion cells?
6. The IPL is formed in between the two pairs of mirror symmetrically arranged cholinergic amacrine cells. Is development of the IPL possible in the absence of cholinergic amacrine cells?
7. Although the cholinergic cells are the main contributors for the development of IPL, early Müller glial cells might render support and stability to the processes from different neurons that stratify the IPL. Can the cholinergic IPL stratifications still form and develop when the Müller glial cells are disturbed?

Chapter I

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Starburst amacrine cells (SACs) develop from a common pool of progenitors which first express AChE very early. ChAT is expressed in the same cells which later differentiate to cholinergic cells and they play major roles during network formation in the IPL. This chapter explains the intricate differentiation path of SACs and their role in IPL development.

Intricate paths of cells and networks becoming “cholinergic” in the embryonic chicken retina

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Abstract

Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are the decisive enzymatic activities regulating the availability of acetylcholine (ACh) at a given synaptic or non-synaptic locus. The only cholinergic cells of the mature inner retina are so-called starburst amacrine cells (SACs). A type-I SAC, found at the outer border of the inner plexiform layer (IPL), forms a synaptic subband `a´ within the IPL, while a type-II SAC located at the inner IPL border projects into subband `d´. Applying immuno-histochemistry for ChAT and AChE on sections of the chicken retina, we here have revealed intricate relationships of how retinal networks became dominated by AChE or by ChAT reactivities. $AChE^+$ cells were first detectable in an embryonic day E4 retina, while ChAT appeared one day later in the very same cells; at this stage all are $Brn3a^+$, a marker for ganglion cells (GCs). On either side of a faint $AChE^+$ band, indicating the future IPL, pairs of $ChAT^+/AChE^-/Brn3a^-$ cells appeared between E7/8. Type-I cells had increased ChAT and lost AChE; type-II cells presented less ChAT, but some AChE on their surfaces. Direct neighbors of SACs tended to express much AChE. Along with maturation, subband `a´ presented more ChAT but less AChE; in subband `d´ this pattern was reversed. Concluding, the two retinal cholinergic networks segregate out from one cell pool, become locally opposed to each other, and become dominated by either synthesis or degradation of ACh. These “cholinergic developmental divergences” may also have significant physiologic consequences.

Introduction

Acetylcholine (ACh) is not a predominant neurotransmitter in the vertebrate retina. However, ACh appears to play not only a decisive role in retinal physiology, but already so during network formation of the inner retina (Baughman and Bader, 1977; Layer, 1983; Marc, 1986; Hutchins, 1987). Cholinergic cells of the inner retina are intriguing, since in all vertebrates they represent a distinct population of amacrine cells (cf. Fig. 1; note: outer retina is not considered here). Three types of cholinergic amacrine cells have been described in the adult chicken retina (Millar et al., 1985, 1987), of which type I and II cells are arranged pair-wise on either side of the inner plexiform layer (IPL; Fig. 1; Prada et al., 1999); due to their morphology they are called starburst amacrine cells (SACs). SACs of type I are located at the INL/IPL border, while type II SACs are displaced to the GCL/IPL border (dSACs; Genis-Galvez et al., 1977; Layer and Vollmer, 1982; Tauchi and Masland, 1984; Masland and Tauchi, 1986; Prada et al., 1999). The rare type III SACs are found deep in the inner nuclear layer (INL), appearing only three days before hatching (Spira et al., 1987). Before segregation of type I and II cells into pairs, SACs in the chicken retina were located in the middle of the emerging IPL (Prada et al., 1999). The processes of type I and II SACs project unilaterally from either side into the IPL, forming two cholinergic synaptic subbands `a´ and `d´ (Fig. 1; Layer et al., 1997; Prada et al., 1999; Drenhaus et al., 2004, 2007; Stanke et al., 2008). Physiologically, they will become involved in retinal OFF and ON pathways, respectively (further, see Discussion). Moreover, since these future cholinergic neurons and their subbands are the earliest to be detected along with the formation of an IPL, e.g. in the chick between E7-E8 (Spira et al., 1987), their formation could actively instruct the formation of others to come (Layer et al., 1997). Based on AChE histochemistry, faint AChE subbands appear very early during retinal development; first as a faint band at E7, which was reported to split into 2 from E9/10 onwards. The very same subbands a and d then become $ChAT^+$. At least two more AChE subbands will emerge which do not overlap with $ChAT^+$ subbands, and thus are non-cholinergic (cf. Fig. 1; Layer et al., 1997). Thus, ample evidence has demonstrated for vertebrate retinae that $ChAT^+$ and $AChE^+$ cells and subbands overlap to some degree, but certainly not fully.

The most reliable marker for cholinergic neurons – not only in retina - is the immunochemical detection of ChAT. On the other side, AChE - as the rate-limiting component of the cholinergic system - is most sensitively detectable by enzyme histochemistry (Karnovsky and Roots, 1964). It has been known since long that AChE labels many more cells than ChAT. In neuroepithelial tissues, e.g. the early retina, AChE becomes strongly expressed in cells shortly after their final mitosis (Layer and Sporns, 1987). Accordingly, in the chicken retina, most cells of the inner half retina express AChE for a certain period of time (Hutchins, 1987; Layer, 1983; Layer and Willbold, 1995; Layer et al., 1997), indicating that all inner retinal cells including ganglion cells would present a major “cholinergic” trait. Yet, not all of them are expected to be (or remain) cholinergic, since in the mature retina only the above mentioned SACs are considered to be cholinergic (on the ambiguity of the term “cholinergic”, see Discussion).

What is the precise spatial and temporal relation between AChE and ChAT expressions in the vertebrate retina? While AChE histochemistry on one side allows to detect AChE activity with utmost sensitivity, on the other side this technique can turn into a drawback, since its brown precipitate can spread over neighboring cells and subbands. This could blur a precise distinction between the localization of both proteins, which prompted us to compare the temporal and spatial appearance and final localization of both populations and their subbands, using improved immunohistochemistry and confocal microscopy, supplemented by RT-PCR experiments. Herewith, we describe most dynamic intricate pathways of the expression patterns of $AChE^+$ and $ChAT^+$ cells of the inner half of the forming and maturing retina. At very early stages, AChE becomes first expressed in a large population of cells, involving most cells of the inner half retina; this includes future ganglion and amacrine cells, which somewhat later also begin to express minor amounts of ChAT. As pairs of $ChAT^+$ cells emerge as a minor part of this primary population, they have lost their AChE expression, while directly neighboring cells further increase their AChE expression. Thus, most conspicuously, each $ChAT^+/AChE^-$ cell has a close-by $AChE^+$ cell(s) directly facing it. A similarly complex pattern is detected during the establishment of the two cholinergic IPL subbands. Eventually in the mature state, a predominance of ChAT over AChE was noted for type I SACs, while for type II SACs this relation was reversed, a novel notion that could have tremendous significance for their physiologic functioning.

Materials and Methods

Animals

Fertilized White Leghorn chicken eggs and adult chicken were purchased from a local farm. The eggs were incubated at 37°C in a humidified chamber under occasional rotation. The stages of development were determined according to the guidelines of Hamburger and Hamilton (1951) and expressed as embryonic days (E) of development. Adult chicken were sacrificed by cervical dislocation. Eyes from different embryonic stages and adult chicken were isolated, washed with phosphate buffered saline (PBS, pH 7.4) and fixed in 4% para-formaldehyde overnight. The eyes were rinsed in PBS and transferred to 25% sucrose overnight. The fixed eyes were immersed in tissue tek (Thermo scientific) and cryosectioned at 10-12µm in a microtome (Microm). The sections were collected on gelatine-coated slides; air dried and stored at -20°C until further use.

Polymerase Chain Reaction (PCR)

The retinal tissues from eyes of embryonic days 4 to 7 were collected and washed with PBS. The tissues were treated with TRI Reagent (Sigma) for RNA isolation. The isolation procedure was according to the protocol supplied by the manufacturer. Briefly, the retinal tissues were homogenized in the presence of TRI reagent by sonication. The homogenate was treated with chloroform to remove protein. RNA was isolated by isopropanol, washed with 75% ethanol, dissolved in DEPC water, and stored at -20°C.

cDNA (complementary DNA) was synthesised using AMV Reverse Transcriptase according to the manufacturer's protocol (Promega). Briefly, the RNA was reverse-transcribed to cDNA in the presence of 25mM $MgCl_2$, 10mM dNTP mix, random or oligo (dT) primer, AMV Reverse Transcriptase enzyme and RNasin (Ribonuclease inhibitor). Reverse transcription was carried out by incubating the mixture at 42°C for 30 min, and inhibition of the enzyme was carried out at 95°C for 5 min and cooled at 0-5°C for 5 min.

PCR was carried out by amplifying the first-strand cDNA in the presence of 25mM $MgCl_2$, 10mM dNTP mixture, 50 picomoles each of both upstream and downstream primers, and 2.5 Units Taq Polymerase enzymes (Bio Labs). Primers were synthesized by using the *Primer3* web based program and ordered from

Euroffins Biotech, Munich, Germany. The primers used were GAPDH sense: *CCTCTCTGGCAAAGTCCAAG*, antisense: *TGGCTGTCACCATTGAAGTC*; AChE sense: *GGTCGAGGGTTCCTATTTCC*, antisense: *ATC-CACGAAGGCCACAATAG*; ChAT sense: *TTGCTGACGACTGATGGAAG*, antisense: *CATGTGCTTGAGCAGGTGTT*. The PCR conditions for GAPDH and AChE were the same with an annealing temperature of 55°C, while that for ChAT was 60°C. The PCR was amplified for 30 cycles for all the primers.

Antibodies

3D10

The monoclonal antibody 3D10 which is also called ACB-1 detects specifically the globular subunit of chicken AChE (Tsim et al., 1988). The asymmetric form of AChE had been isolated from one day-old chick muscle, with which mice were immunized. Western or immunoblotting analysis identified a 110 kDa AChE subunit and showed immunological identity of reaction with all forms of AChE from both brain and muscle. In chick retina, the antibody recognized both extra- and intra-cellular AChE in different types of amacrine cells (Reiss et al., 1996).

ChAT

A polyclonal antiserum against choline acetyltransferase was raised by injecting affinity-purified ChAT obtained from 7-8 week old chick optic lobes by homogenization (Johnson and Epstein, 1986). SDS polyacrylamide gel electrophoresis and nitrocellulose blotting identified a protein of approximately 64 kDa. Its specificity towards ChAT was confirmed by immunoblotting and pre-adsorption assays. In chick retina, the polyclonal antibody recognized ChAT specifically in cholinergic amacrine cells and their processes in two sub layers of the IPL (Millar et al., 1985; Reiss et al., 1996; Prada et al., 1999).

Brn3a

Antibodies against ganglion cell specific Brn3a were kindly gifted by Dr. Eric Turner. The antibodies were prepared by fusing an 80 amino acid region of mouse Brn3a fragment containing sequences N-terminal to the POU specific domain that are not conserved across the members of the other Brn3 family genes (GenBank: AAU13951.1) with glutathione S-transferase fusion vector (Turner et

al., 1994). The bacterial protein was expressed and affinity purified. Western blotting studies from protein extracts from mouse trigeminal ganglia revealed a major band of 43 kDa, consistent with Brn3a cDNA open reading frame (Fedtsova and Turner, 1995). The fusion product was immunized in guinea pigs and the Brn3a antiserum was affinity purified (Quina et al., 2005). In chick, the antiserum specifically recognized ganglion cells of the retina (Dr. E. Turner, personal communication).

Immunohistochemistry

Fixed retinal sections were subjected to immunostaining in order to follow and compare the development and differentiation of cells expressing cholinergic markers in the inner half of the retina. Prior to staining procedures, the frozen sections were dried on a heating plate at 37°C. The area around the sections was marked with a greasy liquid blocker and the slides were pre-incubated in blocking solution (PBS, 3% BSA and 0.1% Triton-X-100) for at least 30 min. 100 µl of primary antibodies diluted in the blocking solution were applied on the sections inside the marked area and then incubated overnight at room temperature. The primary antibodies were applied as mixtures containing one monoclonal and one polyclonal antibody. For AChE and ChAT double staining, the monoclonal antibody 3D10 against AChE and the polyclonal antibody against ChAT (see above), were applied as a mixture together in a dilution of 1:200 and 1:1000, respectively. For Brn3a and ChAT double staining, both the antibodies were applied in a dilution of 1:1000.The slides were washed thrice with PBS and then subjected to 100 µl of fluorescently conjugated secondary antibodies for 70 min at room temperature. Commercially obtained secondary antibodies Cy3 and Cy2 (Dianova) against monoclonal and polyclonal antibodies, respectively, were used in a dilution of 1:200. After three PBS washes, the sections were treated with DAPI for 3 min in order to stain cell nuclei. The slides were rinsed in distilled water after a final wash in PBS and then dried on a heating plate and embedded in Kaiser's glycerol gelatine (Merck, Darmstadt, Germany).

Microscopy and Imaging

Immunostained retinal sections were subjected to Spinning disc Confocal Laser Scanning (Leica) and fluorescent microscopy (Axiophot, Carl Zeiss, Germany) which was connected to an automated digital camera (Axiocam). Imaging was done for each fluorescent channel separately and processed through extended focus imager. All the pictures from different channels were combined as overlays and stored in zvi image format by the Axiovision Basis software program. Confocal images were processed by using ImageJ software. Brightness and contrast was adjusted with Adobe Photoshop.

Results

AChE is expressed before ChAT in the same cells of the early retina

In the mature inner retina, the only cholinergic cells are SACs (see Introduction; Fig. 1; further details see below). Are they the only and first cholinergic cells in the developing chick retina? We have applied the AChE-antibody 3D10 and an anti-ChAT antiserum onto cryosections of E4-5 chicken retinae (Fig. 2a-i) in order to demonstrate that AChE is expressed before the same few cells begin to express ChAT. At this early stage, the neural retina represents a wide neuroblastic layer of cells.

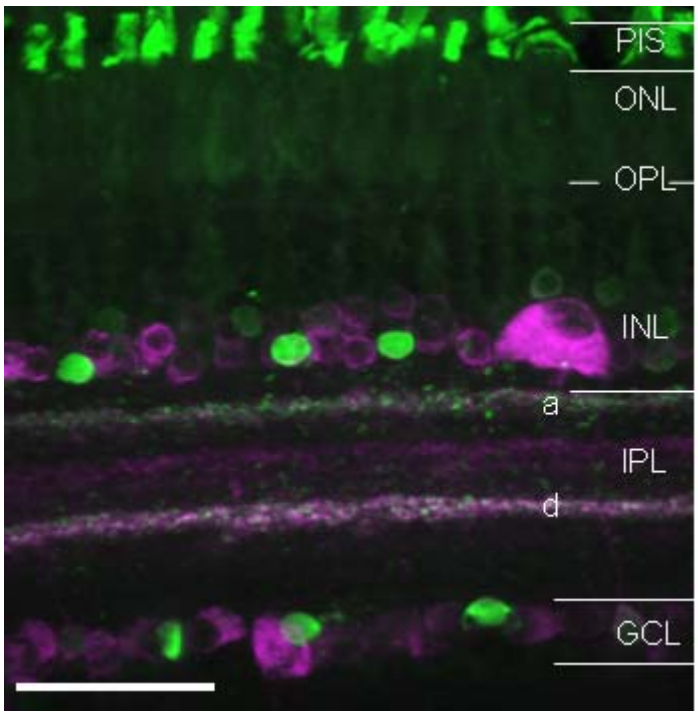

Fig. 1: ChAT- (green) and AChE- (magenta) immunostaining of a cryosection of a mature chicken retina. ChAT-stained cells of the inner retina represent cholinergic starburst amacrine cells (SACs). Two major cholinergic subbands in IPL (a, d) are stained by both, thereby ChAT is stronger in subband `a´, AChE is stronger in subband `d´ (cf. Fig. 8e). Two minor subbands in between are $AChE^+$, but $ChAT^-$ (cf. Layer et al., 1997). Note that stained $ChAT^+$ and $AChE^+$ cell bodies in inner retina are closely juxtaposed, but with little overlap (cf. Fig. 8f), forming 1-2 narrow cell rows in INL and in GCL. Strong ChAT staining in photoreceptor inner segments (*ellipsoids* of ONL) is not subject of this study. Bar = 50 µm. The colors green and magenta for ChAT and AChE staining, respectively, will be applicable for all following figures. Abbrev: GCL, ganglion cell layer; INL, ONL, inner and outer nuclear layers; IPL, OPL, inner and outer plexiform layers; PIS, photoreceptor inner segments.

At E4, only at central locations a small number of cells located at the inner vitreal surface had begun to express AChE (Fig. 2b-c). The $AChE^+$ cells were oval and

slightly bigger in size than their neighbors. At E4, almost none of the cells were stained by the ChAT antibody (Fig. 2a). One day later (Fig. 2d-i), increasingly more cells from center to periphery expressed quite strongly AChE (Fig. 2h, i). Based on their staining intensity, different types of AChE$^+$ cells could be noticed. The more strongly AChE-stained cells in the GCL were bigger in size, as compared to moderately stained cells.

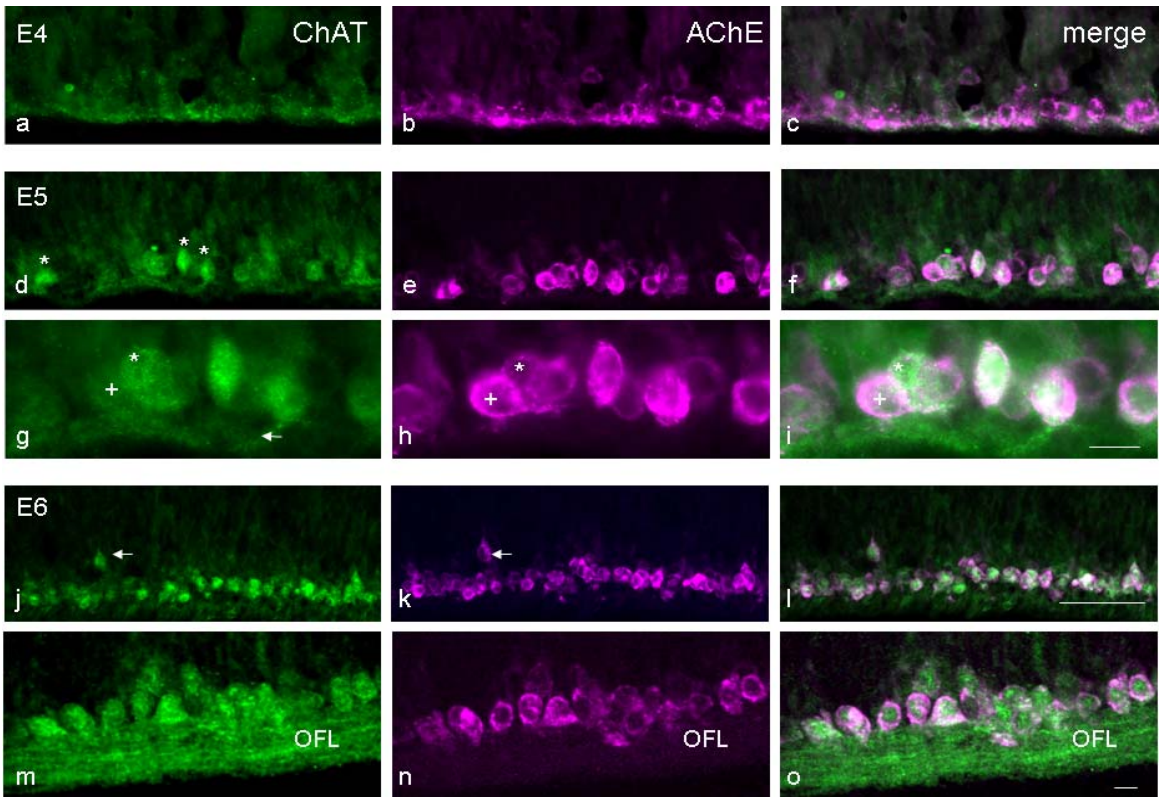

Fig. 2: All innermost retinal cells of E4 (a-c), E5 (d-i) and E6 (j-o) become first AChE$^+$ and then ChAT$^+$, as shown by confocal pictures of immunostained inner retinae for ChAT (left, green), AChE (middle, red) and both (right, merged). The retina is a mere neuroblastic tissue at these stages of development. At E4, AChE$^+$ cells occupied a narrow row adjacent to the vitreal side, while ChAT is undetectable. (d-i): at E5, AChE expression has increased in many cells (see also text), of which only some have become ChAT$^+$ (stars in d); note that AChE is pronounced under cell surface, while ChAT is cell-internal (f and i); (g-i): high magnification pictures of cells from E5. ChAT staining is not yet intense in cells, but note two type of cells among which one cell is weakly reactive for ChAT but strongly AChE reactive (+ in g-i), while the other is vice versa (shown in star in g-i). A mild ChAT staining in the future optic fiber layer (arrow in g), is seen which is completely devoid of AChE. (j-o): At E6, many cells are both AChE$^+$ and ChAT$^+$, and are closely arranged in two irregular rows. Note strong ChAT, but no AChE staining in future optic fiber layer (m, o). Note a double-stained cell migrating towards the vitreal side (arrow). Bar = (a-f and j-l, 50 µm; g-i and m-o, 10 µm).

Only at E5, some of the AChE$^+$ cells now showed also significant ChAT immunostaining (Fig. 2d, g, merge in Fig. 2f, i). Thereby, ChAT staining was more pro-

nounced in the cell interiors, while AChE staining was strong near their surfaces (Fig. 2f, i). Occasionally, cells with quite high ChAT, but low AChE staining could be detected (Fig. 2g-l, star), directly opposed by cells with reversed properties (Fig. 2g-i, cross). A mild ChAT-like immunoreactivity was seen distributed in the forming optic fiber layer (Fig. 2g, arrow). ChAT expression, which at this stage had never been reported before, was confirmed by PCR (Fig. 3a).

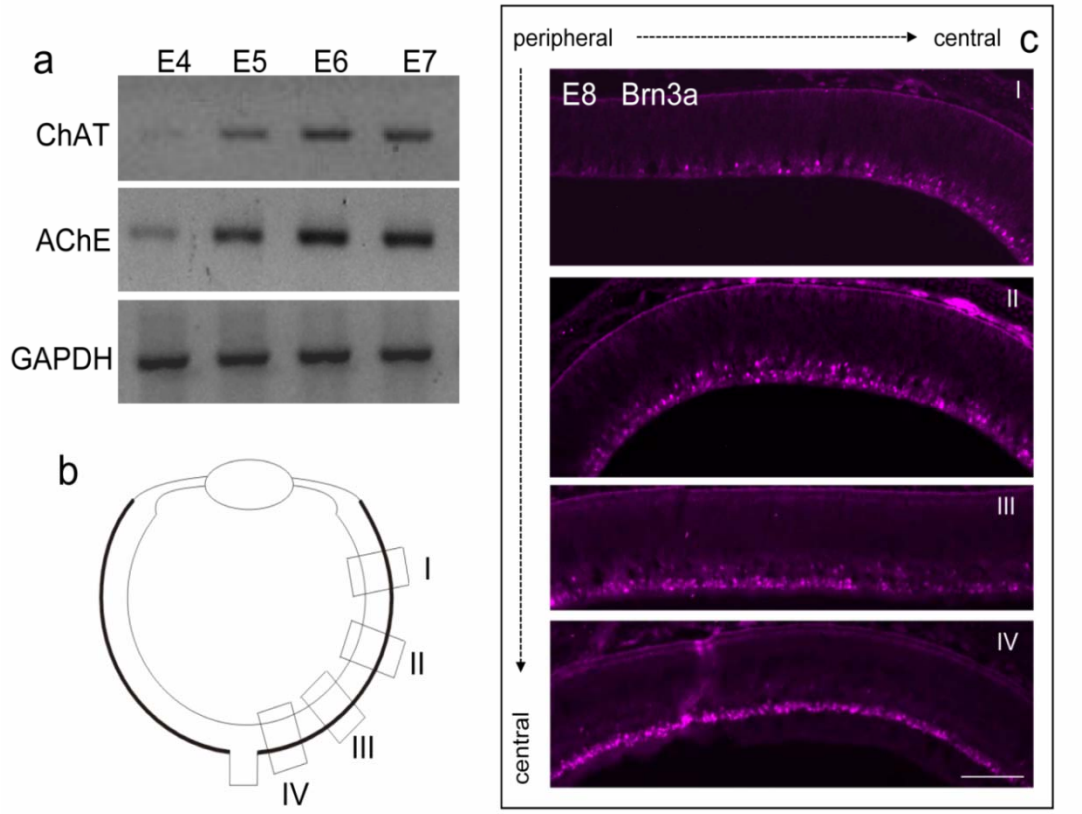

Fig. 3: (a) **Gene expressions for ChAT (lane 1) and AChE (lane 2),** as revealed by PCR from homogenates of E4-E7 chick retinae. At E4, AChE expression is already detected, while ChAT is still almost absent; ChAT gradually increased from E5 onwards. Both expressions reach high levels by E7. Lane 3 shows the expression of GAPDH for control. Further details see Materials and Methods. (c) Settling of Brn3a$^+$ ganglion cells on the vitreal surface from center to periphery. Four regions from an E8 retinal cryosection are presented (cf. Fig. 3b, scheme on left), from a most peripheral sector (I) to sequentially more central sector (II-IV), whereby left sides are more peripheral than the right sides (arrows). Note a second row of Brn3a$^+$ cells emerging near a most peripheral sector (I); these cells are most numerous in section II, while in sector III most, and finally in IV all of them have assembled at the innermost retinal surface. Bar = 100 µm.

At E6, the number of ChAT$^+$ cells had increased and appeared to be distributed in two compact, irregular rows in the central retina (Fig. 2j). At this stage, co-localization of both proteins in all cells was almost complete (Fig. 2l, o). Noticea-

bly at E6, the now wide optic fiber layer was strongly immunoreactive for ChAT, while AChE was entirely absent there ("ofl" in Fig. 2m-o).

Segregation of ChAT$^+$ and Brn3a$^-$ cells and emergence of an AChE$^+$ sub-band, announcing the formation of the IPL

We used the ganglion cell-specific marker Brn3a to follow the fate of future ganglion cells (Fig. 3c, 4) in comparison to ChAT$^+$ cells (Fig. 4). As documented for four different sectors of an E8 retina in Fig. 3c (I-IV indicate different locations from the retinal periphery to the center, see scheme in Fig. 3b), all ganglion cells are assembled near to the vitreal surface. However, in the more peripheral sector a second row of Brn3a$^+$ cells should be noted, which had entirely moved internally in the most central sector IV (see also Fig. 4k, l). Noticeably at E5 to E6, all ChAT$^+$ cells expressed the ganglion cell-specific marker Brn3a (Fig. 4a-f), documenting that early ganglion cells present both major cholinergic properties AChE and ChAT. Only by E7, a segregation of Brn3a and ChAT staining became discernible (Fig. 4g-i). Most Brn3a$^+$ cells (but not all, see Fig. 4h, I, stars) now had settled near the vitreal surface (Fig. 4i). Some of them were still weakly ChAT$^+$. Noticeably, there were also groups of more strongly stained ChAT$^+$ cells which were Brn3a-negative (Fig. 4h, i; circle).

In fact, E7-8 was the time when pronounced cell migrations and segregations led to a cell soma-free space near the internal face of the retina (Fig. 5a). Thereby, the remarkable reorganization of ChAT$^+$ cells (see Fig. 4) was accompanied (or even preceded) by the expression of AChE in the cell soma-free space (Fig. 5b, d). At E7, the more strongly stained AChE$^+$ cells were found along both sides of this cell soma-free space. Announcing the formation of the future IPL, a thin but significant band of AChE-staining became detectable (cf. arrow in Fig. 5b with Fig. 5a). At E7, many cells were ChAT$^+$, most of them also expressing AChE (Fig. 5c, d); only very few were ChAT$^+$ but showing no AChE; these were located within the future IPL (2 arrows in Fig. 5c, d; cf. with 5a, arrow in b). By E8, pairs of ChAT$^+$ cells were arranged pair-wise on either side of the future IPL (Fig. 5g, see also Fig. 4j, l). Noticeably, their AChE expression had become negligible (Fig. 5f). However, the cell soma-free space had become broad, all filled with a band of pronounced AChE expression (Fig. 5e, f, h). This AChE band filled the entire distance between the ChAT$^+$ cell pairs (Fig. 5h), thereby precisely separat-

ing the two rows of SACs. The ChAT$^+$ cells were orderly arranged in two rows, now representing type I and type II starburst amacrine cells, respectively. Type I cells, located at the INL/IPL border, and type II cells, located within the IPL (Fig. 5e, g), both had similar morphologies and had no visible processes, at least as judged by their strong ChAT immunostaining. Except these ChAT$^+$ cells which were AChE-negative, otherwise the number of AChE$^+$ cells had increased, including nearly all cells of the inner half of the INL and most cells of the GCL (Fig. 5f). A quite abrupt decrease of AChE staining towards the outer retina may be noticed (Fig. 5f and h, broken lines).

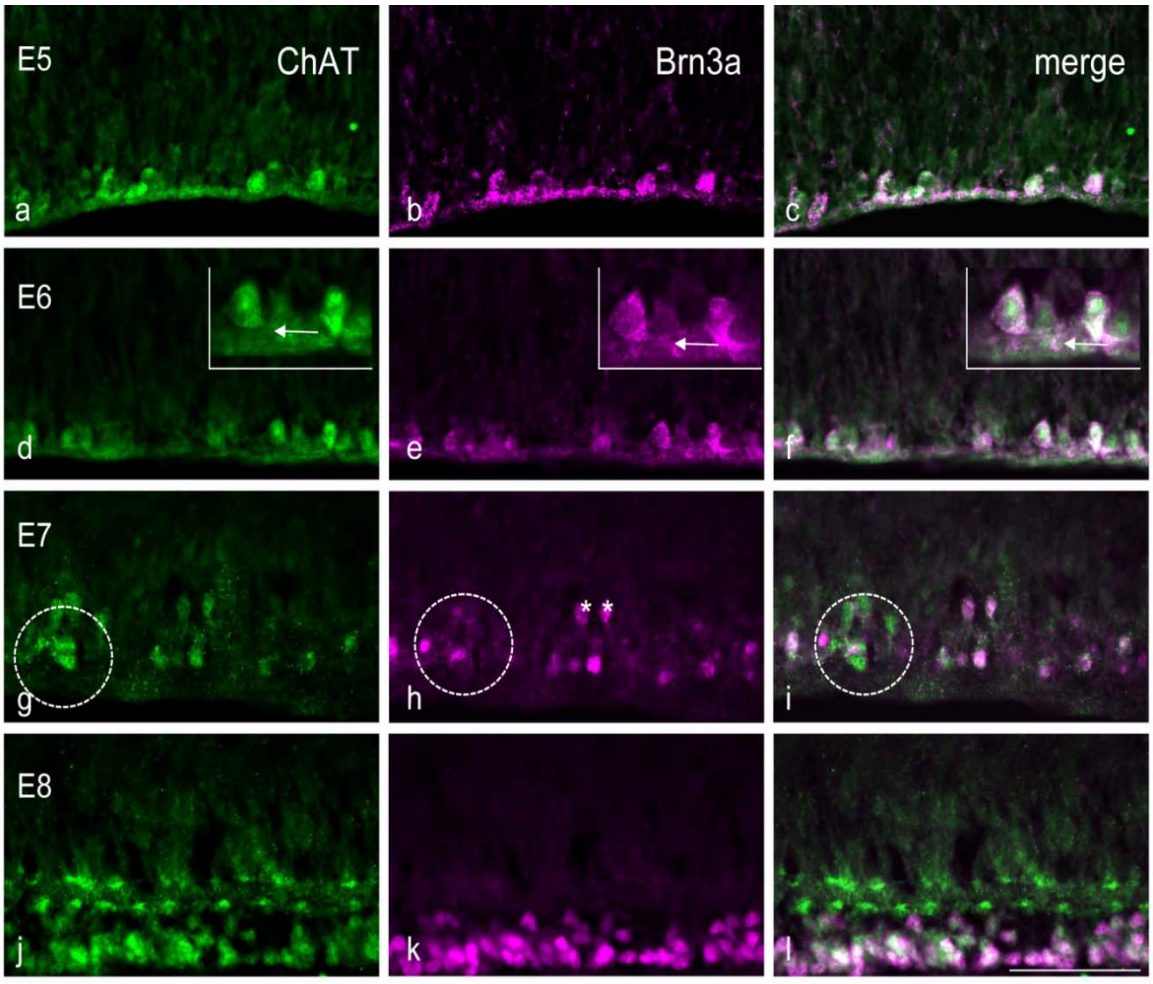

Fig. 4: At E5-6 (a-f), all ChAT$^+$ cells (green) are also stained by the ganglion cell marker Brn3a (magenta). Higher magnification of cells co-localizing ChAT and Brn3a are shown in inserts (d-f). A mild immunoreactivity for both proteins was noticed in the future optic fiber layer (d-f, arrows in inserts), which was reduced by E7 (g-i). By E7 to E8 (g-l), not all ChAT$^+$ cells are still Brn3a$^+$ (shown in circles in g-i). At E7 (g-i), both single- and double-stained cells are interspersed. Note a few Brn3a$^+$ cells which have not yet migrated to the GCL but contain some ChAT (stars in g and h).By E8 (j-l), type I and type II SACs are clearly identified in the IPL by their exclusive ChAT expression, and Brn3a$^+$ ganglion cells are separated from them, which still express some ChAT. Bar = 50 µm.

Formation of two prominent cholinergic IPL subbands

By E9, the IPL had become much wider, and type I and type II SACs were further separated from each other. Their pair-wise arrangement became now highly ordered in space. The cell bodies of type I cells were half embedded into the INL, while their lower half reached into the IPL (Fig. 6a, b). Type II SACs migrated further vitreally, resulting in a broadening of the IPL (Fig. 6a, b). Both cell types formed vertically oriented ChAT$^+$ processes into the IPL, which at a certain distance from their cell bodies turned horizontally (see Fig. 6f). Thereby, both cell types became connected to their neighboring cells, thus forming two ChAT$^+$ neuritic IPL subbands (Fig. 6b, d, f). By E9, both ChAT$^+$ subbands were still separated by a weak AChE$^+$ band (Fig. 6d), the intensity of which decreased from now onwards (Fig. 6f, Fig. 7).

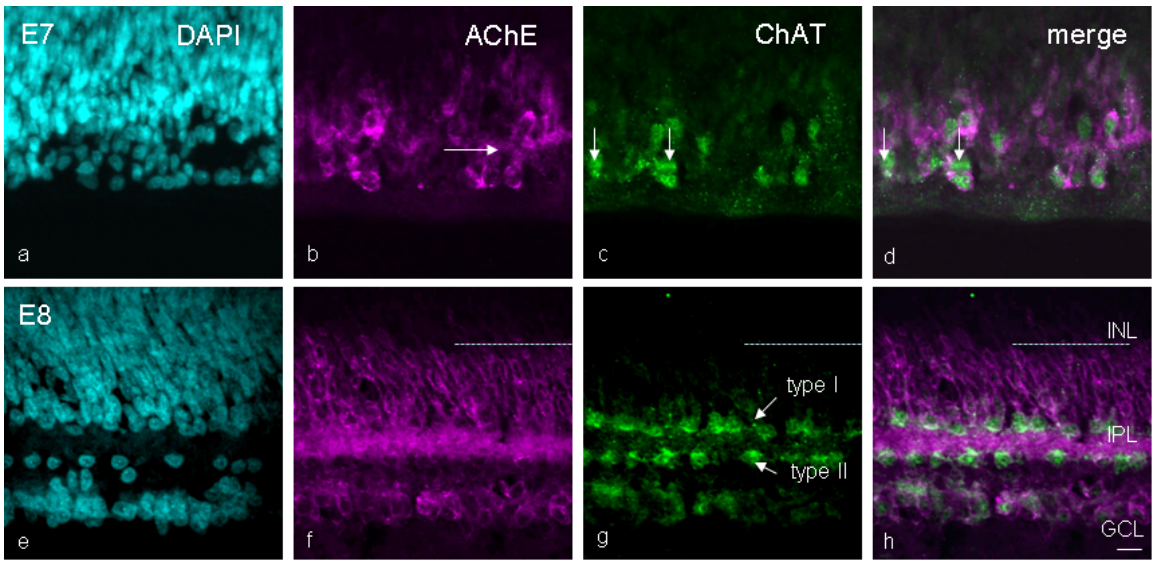

Fig. 5: A new order of AChE and ChAT expression emerges at E7 (a-d) to E8 (e-h) of retinal development, as shown by confocal micrographs for DAPI staining (a, e), AChE in (b, f), ChAT in (c, g), and the latter two rows merged in (d, h). (b, d) at E7, out of many AChE-stained cells, some more strongly stained cells stand out, in between of which a narrow extracellular band of AChE appears (arrow in b), filling a cell soma-free space in (a). ChAT immunoreactivity is most pronounced in cells located in center of future IPL (2 arrows in c, d), and which are the only AChE-negative cells (cf. c with d); (e) at E8, DAPI shows the position of ganglion and a row of displaced amacrine cells (type II SACs) within the forming IPL. (f) Almost all cells of the inner half retina are AChE$^+$; the AChE band in IPL has become broader (f); (g) ChAT$^+$ cells are now clearly depicted in two rows of cell pairs, precisely separated from each other by the AChE band (h). Note also that ChAT in OFL now has disappeared (cf. c, g with m and o of Fig. 2). Bars = 20 µm.

Around E9.5-10, DAPI staining showed that the type II SACs further migrated towards the GCL (Fig. 6d, f), a feature most pronounced towards the optic nerve head (Fig. 6e). Along with it, a differential pattern of ChAT expression was

noticed in different regions of the retina. At a mid-peripheral region, both type I and II SACs expressed ChAT equally, as observed by the intensity of immunoreactivity in both IPL subbands (Fig. 6d). At a region near the optic nerve head, migrating type II SACs lost some of their ChAT expression, while type I cells remained intensely stained (cf. Fig. 6f).

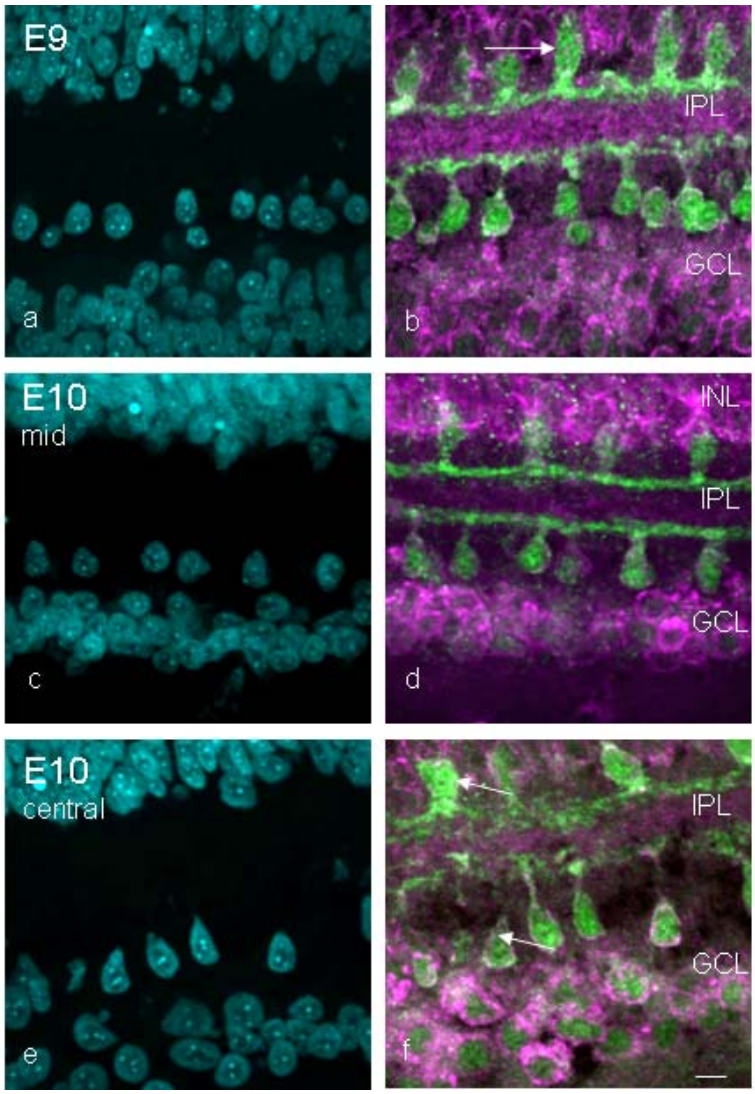

Fig. 6: Differentiation of future cholinergic subbands in the IPL at E9 (a, b) and E10 (c-f). (a, b) At E9, pairs of starburst amacrine cells, one of them at the INL/IPL border designated as type I, and the other near GCL designated as type II, extend processes that are connected with processes of their neighboring cells, thereby forming two continuous IPL subbands, the one near the INL called 'a', the other 'd'. Compared with earlier stages, AChE is decreased and expressed on surfaces of ganglion cells (b), but still separates the $ChAT^+$ cell pairs from each other (b). The type I SACs near the INL are embedded into the INL with part of their cell body reaching into the IPL (arrow in b). (c, d) show retinae near the peripheral region at E10. ChAT staining is predominant in paired cells. The two $ChAT^+$ subbands are stained at equal intensity; the type II SACs are in close vicinity to subband'd' in the inner part of the IPL. (e, f) show retinae at E10, close to the optic nerve. Type II cells have moved further towards the GCL, exhibiting a slight reduction of ChAT immunoreactivity (arrows in f). Note weaker AChE between $ChAT^+$ subbands, and some AChE surface staining on type II, but not on type I cells (f). Bar = 10 µm.

Differential expression of ChAT and AChE in cholinergic IPL subbands

From E12 onwards, the period of synaptogenesis is initiated (Daniels and Vogel, 1980; Hering and Kröger, 1996, Drenhaus et al. 2007). Along with it, a highly differential expression pattern of both ChAT and AChE was noticed. At E12, the IPL still became wider and type II cells remained closely associated with the ganglion cells (Fig. 7a, b), while the two ChAT subbands (called subbands `a´ and `d´) kept their original distance. As a consequence, the vertical processes of type II cells towards subband `d´ became longer (Fig. 7b). From E12 onwards, ChAT staining of type I cells and subband `a´ remained strong, while that of subband `d´ became weaker (Fig. 7b, d. See also Fig.8). Interestingly, as the type II cholinergic cells migrated towards the GCL, the intensity of ChAT immunoreactivity was reduced in subband `d´, while both type I cholinergic cells and subband `a´ remained intensely stained. To further support the differential expression of ChAT in type I versus type II cells, figure 8 presents a wider field of an E12 retina cryosection, immunostained for ChAT. Clearly, ChAT expression is much stronger in type I SACs and in subband `a´ than in type II SACs and subband `d´. The difference in intensity of staining was documented when the pictures were subjected to maximum threshold to produce binary images.

The AChE staining was remarkably different from ChAT staining. As noted above, $AChE^+$ cells occupied a large area in the INL and GCL, and different types of immunoreactive cells could be recognized based on their size and intensity of staining. At E13, the type II cholinergic cells had migrated still further, almost reaching the GCL. The type I cells were strongly stained for ChAT but presented the least intensity of AChE immunoreactivity (Fig. 7d, e-g, stars). The processes extending from type I cells and subband `a´ also had intense ChAT staining. In contrast, type II cells had less ChAT (Fig. 7d, h-j), the protein being localized intracellular, while their membranes were strongly stained for AChE, extending throughout their processes and reaching into subband `d´ (star in Fig. 7i). In the INL, each type I cholinergic cell was neighbored by a non-cholinergic amacrine cell that was strongly immunoreactive for AChE (Fig. 7d, wide arrows). At E14, this expression pattern of both enzymes had not much changed, but the stronger expression of ChAT on the INL side including subband `a´ had become even more pronounced (not shown).

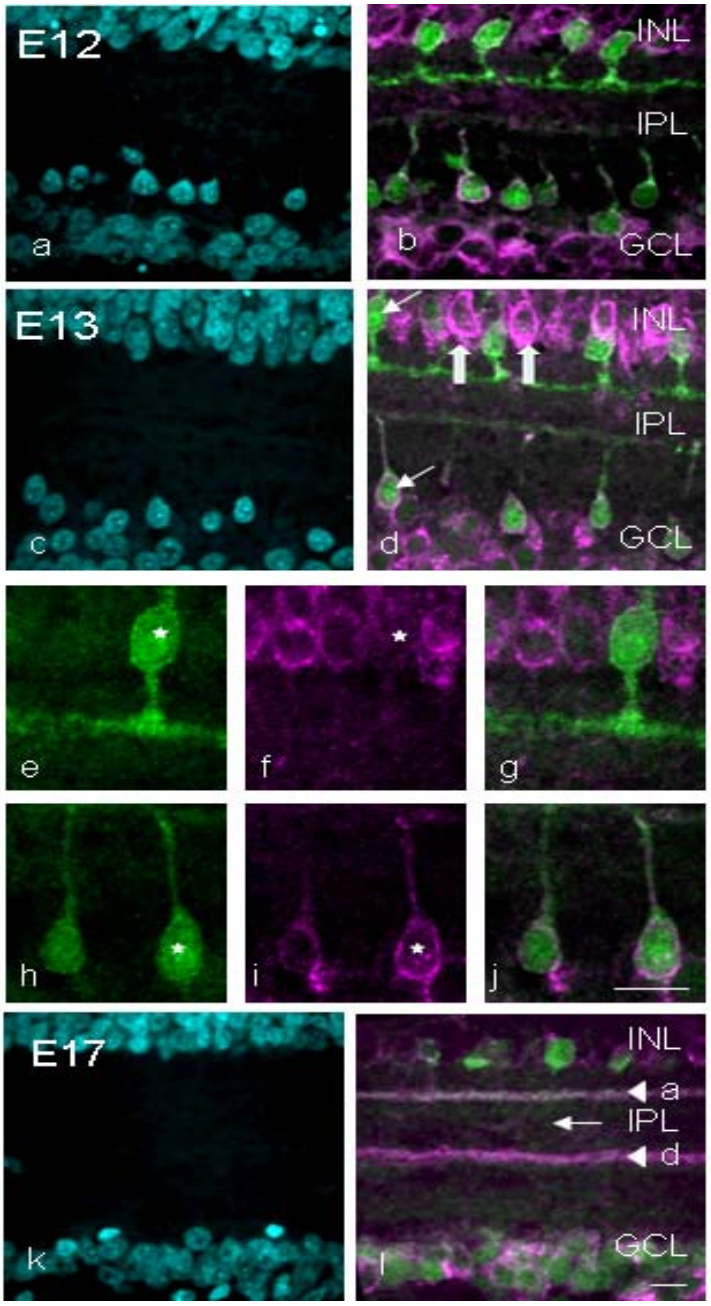

Fig. 7: Differential expression of both ChAT and AChE in type I and type II cholinergic amacrine cells, and in their neighbors during periods of synaptogenesis. (a, b) show expression of ChAT and AChE at E12. DAPI staining reveals further migration of type II cells towards the GCL (a). Note reduction of ChAT immunoreactivity in subband'd', as type II cells migrate away from it (b). AChE is strongly expressed in cells neighboring $ChAT^+$ cells; weak AChE in the IPL now coincides with $ChAT^+$ subbands (b). (c, d) show E13, now with increased width of IPL. Type II cells are located within ganglion cells. Note strong surface AChE with no ChAT staining of INL cells next to type I cells (d, wide arrows); type II, but not type I cells show AChE surface staining (d, small arrows). (e-j) high magnification confocal micrographs of type I and II cells (marked by arrows in d) show different expression patterns of both proteins. Note a high content of ChAT in a type I cell in the INL (e, star), while it has reduced AChE content (f, star; g is merge of e, f). (h-j) show type II cells which in comparison to type I cells has less ChAT content, but increased AChE surface staining and along its process (stars in h, i). (k, l) show E17, at which stage the AChE staining in cell bodies has somewhat decreased, while AChE staining of subbands has become much stronger, particularly in subband d. Bars = (a-d, k, l, 20 µm), and (e-j, 10 µm).

At E17, the IPL had become much broader. Type II cholinergic cells had reached the GCL and were integrated among ganglion cells. By then, these cells displayed very weak ChAT immunoreactivity, and the corresponding subband in the IPL was also weakly stained (Fig. 7l). At this time, both subbands were strongly immunoreactive for AChE, but still subband `a´ was dominated by ChAT and subband `d´ by AChE (Fig. 7 l) As the width of the INL was reduced, $AChE^+$ cells of the INL were reduced considerably to 2-3 cell layers (cf. Fig. 1). In the IPL, an additional $AChE^+$ subband was detected which was ChAT-negative (Fig. 7l, arrow; Fig. 1).

In the adult chick, the whole retinal tissue had become thinner compared to the embryonic stages (Fig. 1). By then, the number of cholinergic cell pairs had drastically decreased; their pair-wise arrangement was not well visible. In type II cells and in subband `d´, ChAT immunoreactivity had again somewhat increased (Fig. 1). The distance between subband `d´ and the GCL was reduced. Interestingly, $ChAT^+$ types I and II cholinergic cells were consistently neighbored by large cells which were intensely stained for AChE (Fig. 1). In spite of the up-regulation of ChAT in type II cells, the predominance of AChE in the latter was still persistent (Fig. 1). In addition to the two main IPL subbands, two further $AChE^+$ subbands which were ChAT-negative were noticed in the IPL (Fig. 1). Thus at adult stages, subband `a´ was dominated by ChAT over AChE, while in subband `d´ the situation was reversed.

Discussion

Although cholinergic cells and properties appeared to be well established for different vertebrate retinae (refs. see Introduction; Baughman and Bader, 1977; Ross et al., 1985; Marc, 1986; Masland and Tauchi, 1986; Millar et al 1985; Millar and Chubb, 1987; Spira et al, 1987; Famiglietti et al, 1983a, 1983b ; Nguyen and Grzywacz, 2000), this detailed study on the comparative developmental expressions of AChE vs. ChAT has revealed several novel aspects which should help to better understand both developmental and physiological processes in the vertebrate retina. This significant progress became possible due to improved immunocytochemical techniques as well as confocal microscopy, using two well established antibodies, e. g., the monoclonal antibody 3D10 against AChE, and the polyclonal rabbit anti-serum against ChAT (Johnson and Epstein, 1986).

AChE precedes ChAT expression in the very same cells

Not relying on AChE histochemistry, which can blur the cellular resolution of results after longer exposure times, we here have used AChE immunohistochemistry to compare precisely the time of AChE vs. ChAT appearance in cells of the chicken retina. Beginning in the central retina, AChE could be detected from E4 onwards, while ChAT appeared in the very same cells approximately one day later. Our detection of ChAT by E5 is in agreement with other immuno- and mRNA analyses (Bader et al., 1978), but occurred somewhat earlier than was reported in another study by E6.5 (Spira et al., 1987), possibly due to improved immuno- and microscopic techniques.

Based on AChE histochemistry, AChE could be detected even somewhat earlier (Layer, 1983). Noticeably, in many parts of the early neural tube AChE expression immediately follows the last mitotic cycle, establishing it as an early post mitotic neuronal marker (Layer and Sporns, 1987; Layer et al., 1988). This and other findings indicated that AChE expression by itself does not establish the respective cells as “cholinergic” (see below, and for review, Layer and Willbold, 1995). Rather, the expression of ChAT is generally accepted to render a cell with this property. That AChE temporally precedes ChAT expression in the very same post mitotic cells could indicate their co-regulation. While their interaction(s) on the genetic and molecular levels remains elusive, their direct or indirect interdependence has been reported repeatedly. For instance, a ChAT promoter region

was found to be related to the response of AD patients to AChE inhibitors (Harold et al., 2006).

Ganglion cells transiently express ChAT during development

During the period of E5-6.5 most $AChE^+$ cells expressed at the same time also ChAT (Fig. 2l). The first produced cell type in the retina are ganglion cells (Cepko et al., 1996; Prada et al., 1991). The majority of the $AChE/ChAT^+$ cells were ganglion cells, as shown by their expression of the Brn3a marker. Thus, this finding establishes that ganglion cells, which in their mature state are supposed to be non-cholinergic, can synthesize and degrade ACh during their early embryonic life.

What is a "cholinergic cell"? Unclear definitions

Does the term "cholinergic" mean any cell that is somehow involved in cholinergic mechanisms, e.g. cells expressing either one or more of the respective components? According to classical textbook definitions, a "cholinergic cell" is a cell using ACh as its neurotransmitter. However, at times the term "cholinergic" is being used for cells synthesizing ACh, or for cells degrading it, or cells presenting receptors or transport proteins for ACh. Hence, the general use of "cholinergic" in the literature is quite diffuse. Conventionally, for the inner vertebrate retina the starburst amacrine cells (SACs) are supposed to be the only cholinergic cells. Our study exemplifies this problem: on the one side, the situation for the mature retina is clear, in that the mature SACs produce ChAT, and low, or negligible amounts of AChE, and thus can be truly called "cholinergic", e.g. they represent ACh-producing cells. Whether early ganglion cells which are similarly $AChE^+$ and $ChAT^+$ can be considered "cholinergic" remains ambiguous. Given this ambiguity, we here have restricted ourselves and call them $AChE^+$ and/or $ChAT^+$ cells.

AChE might initiate the separation of type I and II SACs as a first step of IPL formation

A most significant change in expressions of both proteins occurred around E7-E8. A second row of Brn3a cells settled within the GCL. These cells still expressed both proteins (albeit at various levels). But quite abruptly, by E7 a single

row (Fig. 6c, arrows), and by E8, a double row of $ChAT^+$, but $AChE^-$ cells (Fig. 6g, arrows) emerged in a cell soma-free space, announcing the future IPL space. Noticeably, these cells - which can now be addressed as future cholinergic SACs - were distinct from, nor did originate from the second row of ganglion cells (mentioned above). Also it is remarkable that these two rows of $ChAT^+$ cells are precisely separated by a wide IPL band of AChE, which has been shown to be exposed extracellularly (Reiss et al., 1996). Further, all cells of the inner half of the INL were $AChE^+$, but $ChAT^-$, with a sharp border towards the outer INL, as has been pointed out earlier by enzyme histochemistry (Layer et al., 1997). As these $ChAT^+$ cells extend processes around E9-E12, these neurites will never cross through this IPL band of AChE activity. We suggest that this IPL band of AChE activity creates a space completely free of acetylcholine, which then may prevent neurites from intruding into this space. Such a possible function of ACh has been corroborated by *in vitro* studies, e.g. ACh could inhibit neurite growth (Lipton et al., 1988), but also could attract them (Zheng et al. 1994), depending on species and culture conditions (Song & Poo, 1997). Moreover, AChE itself could direct neuritic growth by non-enzymatic, adhesive mechanisms (Layer et al., 1993; reviewed in Scholl and Scheiffele, 2003; Vogel-Höpker et al., 2011). In other words, the $AChE^+$ IPL band could be responsible for spatially organizing the future two cholinergic IPL subbands. This conclusion is strongly supported by in vivo findings in an AChE knockout mouse, where the formation of ordered cholinergic and other IPL subbands was disturbed (Bytyqi et al., 2004; also own observations).

Non-cholinergic $AChE^+$ cells neighbor each type I SAC in the INL

As the period of synaptogenesis in the IPL approached its end from E13 to E17, the pattern of AChE vs. ChAT expressions underwent further intricate transformations (Fig. 7). On the INL side, the general expression of AChE in cells decreased, but leaving a row of most strongly AChE expressing cells (e.g. Fig. 7d, wide arrows). In fact, among the $AChE^+$ cells on the INL side five different types of cells could be distinguished, based on their dendrites ending in the different cholinergic and non-cholinergic IPL subbands a-d (Layer et al, 1997; cf. Fig. 1). Consistently, $AChE^+$ cells on the INL/IPL border neighbored each type I cholinergic amacrine cell. Sitting somewhat further inside the INL, in these cells the AChE appeared directly near the cell surface, but had been reported to be not accessi-

ble from the exterior of the cell (Reiss et al., 1996). It seems that AChE in these cells could provide an entirely ACh-free interior within the cells, the role of which remains elusive (e.g. co-regulate cell proliferation, differentiation, etc.). The $ChAT^+$ cells (type I SACs) were completely free of AChE. Their ChAT appeared throughout the entire cells, including their processes, which were nearly free of any AChE. It would be most interesting to know whether $ChAT^+$ cells can release ACh from their cell bodies and from their processes, and possibly have a cholinergic cross-talk with their neighboring $AChE^+$ cells, similar to the situation at cholinergic synapses. To further elaborate on this, expression studies for different types of ACh receptors need to be included.

On the side of the GCL, the situation was distinctively different. Type II SACs expressed lesser ChAT, but still showed AChE on their surfaces. Both proteins were also easily detectable in, or on their long vertical processes into subband d. The ganglion cells expressed both proteins at various levels. Type II cells were not directly neighbored by cells with extremely strong AChE expression (as were type I cells).

Differences between type I and type II SACs

Thus in this study, one of the striking findings were the notion of increasing differences between the two cholinergic proteins on either side of the developing IPL. The formation of the IPL with two major cholinergic subbands has attracted much attention recently. IPL differentiation based on NgCAM and axonin-1-immunoreactivity along with synaptogenesis was reported to begin only at E9, eventually forming five subbands by E14 (Drenhaus et al., 2004). Interestingly, already at E7 an $NgCAM^+$ subband announced the future IPL, corresponding fully with our faint $AChE^+$ subband in the future IPL at this stage (Fig. 5). Well supported by our present study, these authors could detect a pair-wise arrangement of $ChAT^+$ SACs by E8, and onset of their process formation first of type I cells on E9, and of type II cells by E10.

It remains a difficult question to localize precisely where the SACs are derived from. As presented in Fig 5c, few $ChAT^+/AChE^-$ cells could be traced in the middle of the future IPL (these were even better resolved at the far-most retinal periphery of much older retinae; data not shown), which shortly thereafter have formed two separate rows (Fig. 5g). It is important to note that this pool of cells is

the first that expresses high ChAT, but negligible or no AChE. These findings correspond well with a detailed Golgi study which could document that the pairs of SACs are derived from a pool of post mitotic cells located in the center of the future IPL. During this period both type I and type II cells were claimed to express ChAT and GABA, until their segregation towards opposite sides occurred (Prada et al., 1999). Since the expression of both neurotransmitters occurs very early, these authors suggested a morphogenetic role for both ACh and GABA, on which we agree, at least as far as ACh is concerned.

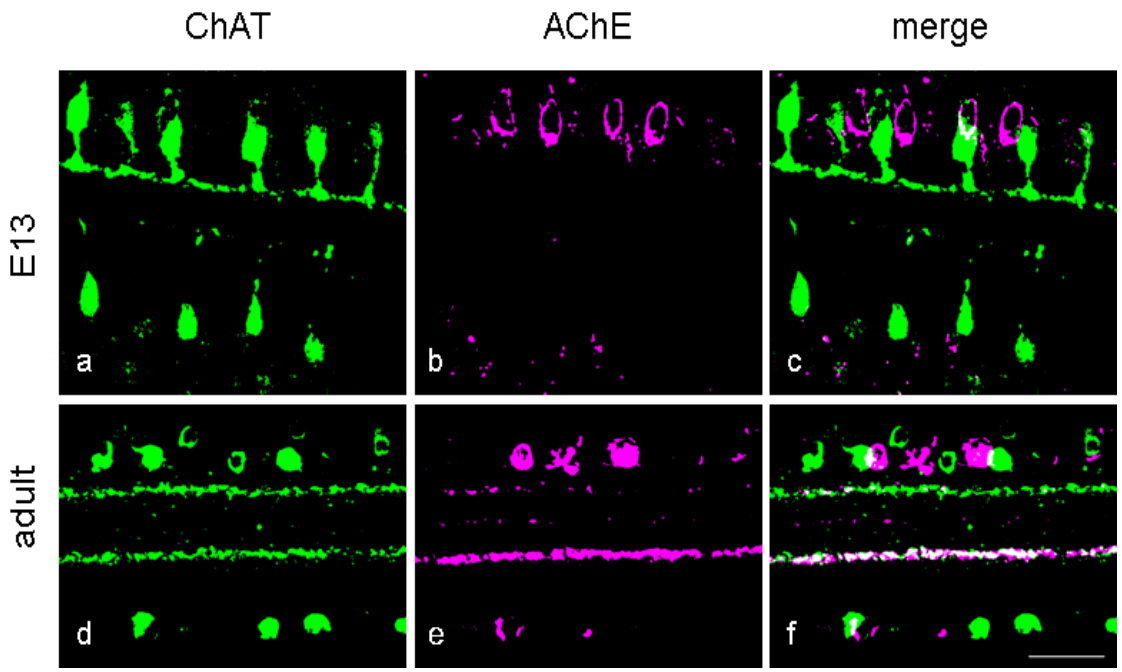

Fig. 8: Image analysis of comparative expressions of ChAT (green) and AChE (magenta) in both type-I and type-II SACs at E13 (a-c) and adult stage (d-f), as achieved by setting the signal threshold for each channel individually such that backgrounds, minor stained cells and subbands were suppressed. Note that ChAT is stronger in type-I SACs and subband `a´ at E13, but not so anymore at adult stage. For AChE, note close association of strongly stained $AChE^{+}$ cells next to type-I SACs (see c), and much stronger AChE staining of subband `d´ at adult stage (see e). (a-c) are derived from Fig. 7c, d; (d-f) are confocal pictures of an adult retina; a merge of both channels is shown in right frames. Bar = 20 µm.

Another significant chicken study established that type I and type II SACs are not identical during their development (Stanke et al., 2008). Type I SACs expressed Pax6, Islet-1 and $p27^{kipl}$, while a sole feature of type II cells during their migration towards the GCL was their expression of cellular retinoic acid binding protein (CRABP) and neurofilaments, corresponding well with our own observa-

tions (not shown). Remarkably, the regular spatial distribution of SACs appeared to depend on muscarinic mechanisms (Stanke et al., 2008).

Adult stage: physiologic consequences of cholinergic differences of type I and II SACs?

Reaching the mature stage in the retina, the distribution of both cholinergic proteins becomes even more distinct, and thus simpler (from Fig. 7k, l to Fig. 1). Being not a topic of this study but to complement the picture from figure 1, the outer parts of the photoreceptor inner segments expressed much ChAT, with no AChE being detectable there. Interestingly, a stress-induced isoform of AChE (*AChE-R*) was induced in light-stressed rat retinae, and was suggested to counteract death of photoreceptors (Kehat et al., 2007). This aspect will need a separate detailed study.

Concerning the inner retina, first it should be mentioned that the highly regular, pair-wise arrangement which we could easily follow from E8 – E11/12, was by far not as evident in the adult retina (Fig. 1), particularly so in more central retinal sectors. It is conceivable that this could be a consequence of intricate network formations within the inner retina.

As the most significant aspect, our findings relate to physiological differences between type I SACs and its subband `a´ as compared with type II SACs and subband `d´. The retinal physiology is balanced by OFF and ON channel pathways which are formed by the processes of type I and type II SACs. Both the channels are formed by complex stratifications of sets of ganglion, amacrine and bipolar cells. By definitions, the ON channel is formed by the subsets of ON ganglion, amacrine and bipolar cells which fire an action potential when their receptive fields are illuminated (bright light), while the OFF channel is responsive to dark and is served by the OFF subsets of cells (Layer et al 1997). Type I cells are part of the so-called OFF channel, while the cholinergic type II system functions within the ON channel (Rodieck, 1998). Our findings have revealed that the cholinergic OFF side of the IPL is more driven by synthesis of ACh, while the ON side is dominated by degradation of ACh. In other words, the cholinergic OFF channel could be governed by an excess of ACh, while the ON channel might experience a constant ACh undersupply. Such a basic difference between the

cholinergic functioning of type I vs. type II SACs has never been observed or discussed before. Without being experts in retinal physiology, we feel it seems logical that these differences might contribute to the functioning of inhibitory cholinergic pathways in the OFF and stimulatory mechanisms of ACh in the ON channel.

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Chapter II

Manuscript in preparation

The differentiation of ganglion cells (GCs) and amacrine cells (ACs) overlap closely. The expression of ChAT as early as E5 at the presumptive GCL leads us to suspect if differentiating GCs express ChAT. This chapter describes that GCs transiently express ChAT and explains their role in the differentiation of the SACs.

Differentiating ganglion cells transiently express choline acetyl-transferase in chick embryonic retina

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Key words: acetylcholinesterase (AChE); Brn3a for ganglion cells; choline acetyl-transferase (ChAT); differentiation; starburst amacrine cells (SACs)

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Abstract

The first cells to differentiate in the vertebrate retina are the ganglion cells (GCs). Differentiation is not a single step process and the cells begin to express different types of proteins at different stages throughout the process. In the present study, chick embryonic retina was used as a model system to follow the fate and differentiation of the GCs. The most accepted marker protein, Brn3a, a POU domain transcription factor which is specific for ganglion cells was used to follow the expression pattern of the same during early embryonic retinal development. Acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) were also used as markers in order to follow the differentiation of cholinergic amacrine cells which begin their differentiation shortly after the GCs. Though ganglion cells are the first to begin their differentiation, they became specified only around E7/E8. At stages around E5/E6, cells that expressed Brn3a co-localized ChAT and CRABP, which are amacrine cell markers. After E7, the same cells upregulated the expression of Brn3a and lost ChAT and CRABP expression as revealed by immunohistochemical staining, thereby specifying themselves to become ganglion cells while another set of closely abiding cells did the vice-versa to become cholinergic amacrine cells. Thus, the ganglion and cholinergic amacrine cells share a common progenitor cell population and during differentiation, the ganglion cells transiently express ChAT.

Introduction

Differentiation is a vital process by which the cells after exiting the cell cycle, express cell-specific genes to become a specialized cell type from a pool of progenitors. The first cells to differentiate during early chick embryo retinogenesis are the ganglion cells (GCs). In chick, thymidine incorporation studies showed that the presumptive GCs withdrew from the cell cycle on late second or early third day of retinal development (Kahn, 1973, 1974). These studies further reported that the GCs are generated between 2 and 5 days of incubation. Extensive studies based on Golgi method emphasizing the migration, development and growth of dendrites from the GCs are available (Nishimura, 1980; Prada et al 1981). Though these techniques are highly useful in tracing the early post-mitotic neurons from ventricular positions to late migratory positions, they are often confusing due to the lack of specificity. The main reason is that the ganglion cells share the ganglion cell layer (GCL) with equal or more numbers of displaced starburst amacrine cells (dSACs; Famiglietti, 1983; Genis-Galvez et al., 1977; Layer and Vollmer, 1982; Tauchi and Masland, 1984).

Alternative methods of identifying differentiating GCs are by in situ hybridization to detect GC-specific mRNAs or immunodetection of proteins specifically expressed by GCs. Early post mitotic and migrating GCs in chick were detected by RA4 antibodies (Waid and McLoon, 1995). In recent times, Brn3a is mostly accepted as a specific ganglion cell marker in different species (Nadal-Nicolas et al., 2009). The Brn3 family of the POU-domain transcription factors has been shown to play major roles in survival, differentiation and dendritic growth of retinal ganglion cells. These factors have been reported to show remarkable structural similarities to the POU-domain transcription factor Unc-86, which is critical for the differentiation of neurons in the nematode C. elegans (Fedtsova and Turner, 1995).

During differentiation, the GCs also express neurofilaments (McCabe et al., 1999), cellular retinoic acid binding protein (CRABP) (Mey et al., 2001), acetyl cholinesterase etc. We used immunohistochemical techniques by employing Brn3a, CRABP, AChE and ChAT (choline acetyltransferase) as markers to distinguish differentiating GCs from other cell types during the early (E5-E8) period of embryonic chick retinal development. We report here that differentiating GCs express ChAT transiently during the early period of retinogenesis (E5/E6) but

immediately stop the expression from E7 onwards and are never ChAT$^+$ throughout development. The possible functions of early ChAT expression by GCs are briefly discussed.

Materials and Methods

Animals

Fertilized White Leghorn chicken eggs were purchased from a local farm and incubated at 37°C in a humidified chamber under occasional rotation. The stages of development were determined according to Hamburger and Hamilton (1951) and expressed as embryonic days (E) of development. Adult chicken were purchased and sacrificed by cervical dislocation. Eyes from adult chick and embryonic stages E5-E8 were isolated, washed with phosphate buffered saline (PBS) and fixed in 4% para-formaldehyde overnight. The eyes were rinsed in PBS and transferred to 25% sucrose overnight. The fixed eyes were immersed in tissue tek (Thermo scientific) and cryosectioned at 10-12µm in a microtome (Microm). The sections were collected on gelatine-coated slides; air dried and stored at -20°C until further use.

Immunohistochemistry

In vivo whole mount sections of adult and embryonic eyes were subjected to immunostaining in order to follow development and differentiation of ganglion cells. Prior to staining procedures, the frozen sections were dried on a heating plate at 37°C. The area around the sections was marked with a greasy liquid blocker and the slides were pre-incubated in blocking solution (PBS, 3% BSA and 0.1% Triton-X-100) for at least 30 min. 100 µl of primary antibodies diluted in the blocking solution were applied on the sections inside the marked area and then incubated overnight at room temperature. The primary antibodies contained the monoclonal antibody against Brn3a, another monoclonal antibody 3D10 against AChE and the polyclonal antibody against Chat, or a mixture of Brn3a, CRABP and ChAT. The dilution factor of the antibodies was as follows: anti-Brn3a-1:1000, 3D10-1:200, CRABP-1:1000 and ChAT-1:1000. The slides were washed thrice with PBS and then subjected to 100 µl of fluorescently conjugated secondary antibodies for 70 min at room temperature. Commercially obtained secondary antibodies Cy3, Cy5 and Cy2 and (Dianova) against monoclonal and polyclonal antibodies, respectively, were used in a dilution of 1:200. After three PBS

washes, the sections were treated with DAPI for 3 min in order to stain cell nuclei. The slides were rinsed in distilled water after a final wash in PBS and then dried on a heating plate and embedded in Kaiser's glycerol gelatine (Merck, Darmstadt, Germany).

Microscopy and Imaging

Immunostained *in vivo* sections were subjected to fluorescent microscopy (Axiophot, Carl Zeiss, Germany) which was connected to an automated digital camera (Axiocam). Imaging was done for each fluorescent channel separately and processed through extended focus imager. All the pictures from different channels were combined as overlays and stored in zvi image format by the Axio-vision Basis software program. Brightness and contrast was adjusted with Adobe photoshop.

Results

Ganglion cells (GCs) are the first to differentiate in any vertebrate retina. In the mature retina, the ganglion cells form a single layer of cells within the ganglion cell layer (GCL) (Fig. 1). They share the GCL with the displaced starburst amacrine cells (dSACs), which are otherwise called type II cholinergic amacrine cells. As GCs express Brn3-family of transcription factors during differentiation, we used Brn3a, a widely accepted marker of differentiating GCs, to follow the differentiation of GCs.

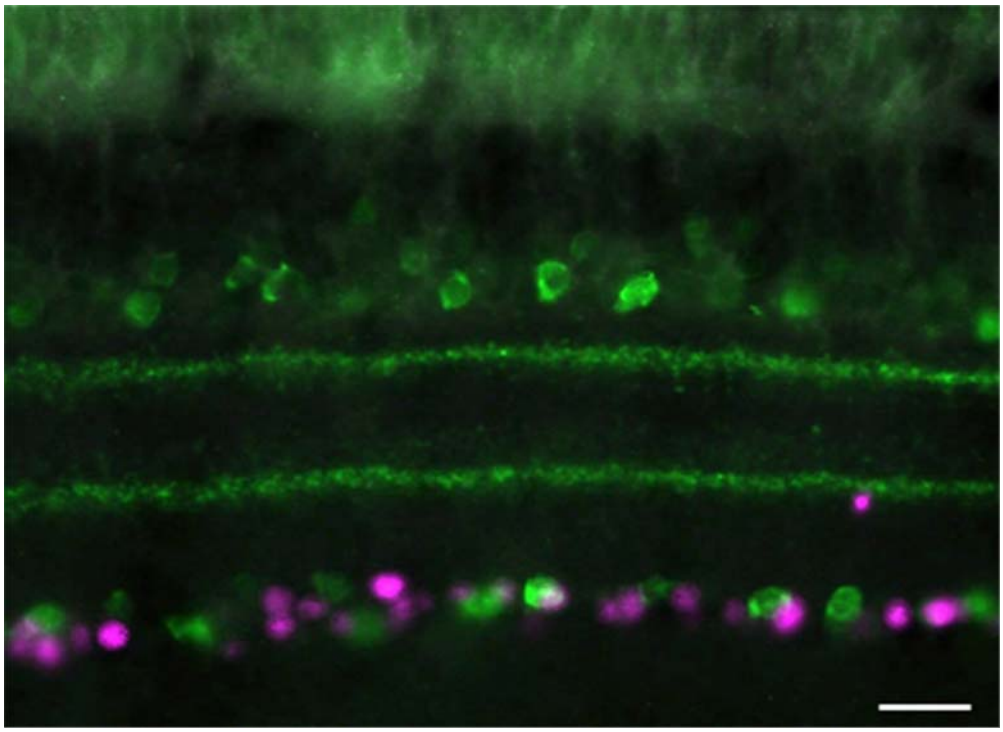

Fig. 1: Expression of Brn3a and ChAT. Section of an adult chick retina immunostained for Brn3a (magenta) and ChAT (green) in order to differentiate between ganglion cells and starburst amacrine cells. Type I and type II cholinergic amacrine cells are clearly visible, their processes end in the IPL, forming two prominent cholinergic subbands. The GCL consists of a single layer of cells shared by both the ganglion and displaced starburst amacrine cells. Note that the staining of Brn3a and ChAT does not overlap, suggesting the specificity of markers towards the two populations of cells. Abbrev: IPL- inner plexifrom layer, GCL- ganglion cell layer, ChAT- choline acetyltransferase. Scale bar = 50 µm.

Expression of Brn3a marks the specification of ganglion cells

The ganglion cells are the first born cells which are followed by photoreceptors, amacrine cells, horizontal cells, bipolar cells and finally Muller glial cells. Though ganglion cells are born around E3 and immediately start differentiating (Kahn, 1973), their time of specification is speculative. In this study we show that

the specification of GCs begins with the onset of expression of Brn3a around E5 at a central locus of the retina (Fig. 2a arrow). These cells were found at the vitreal surface of the retina.

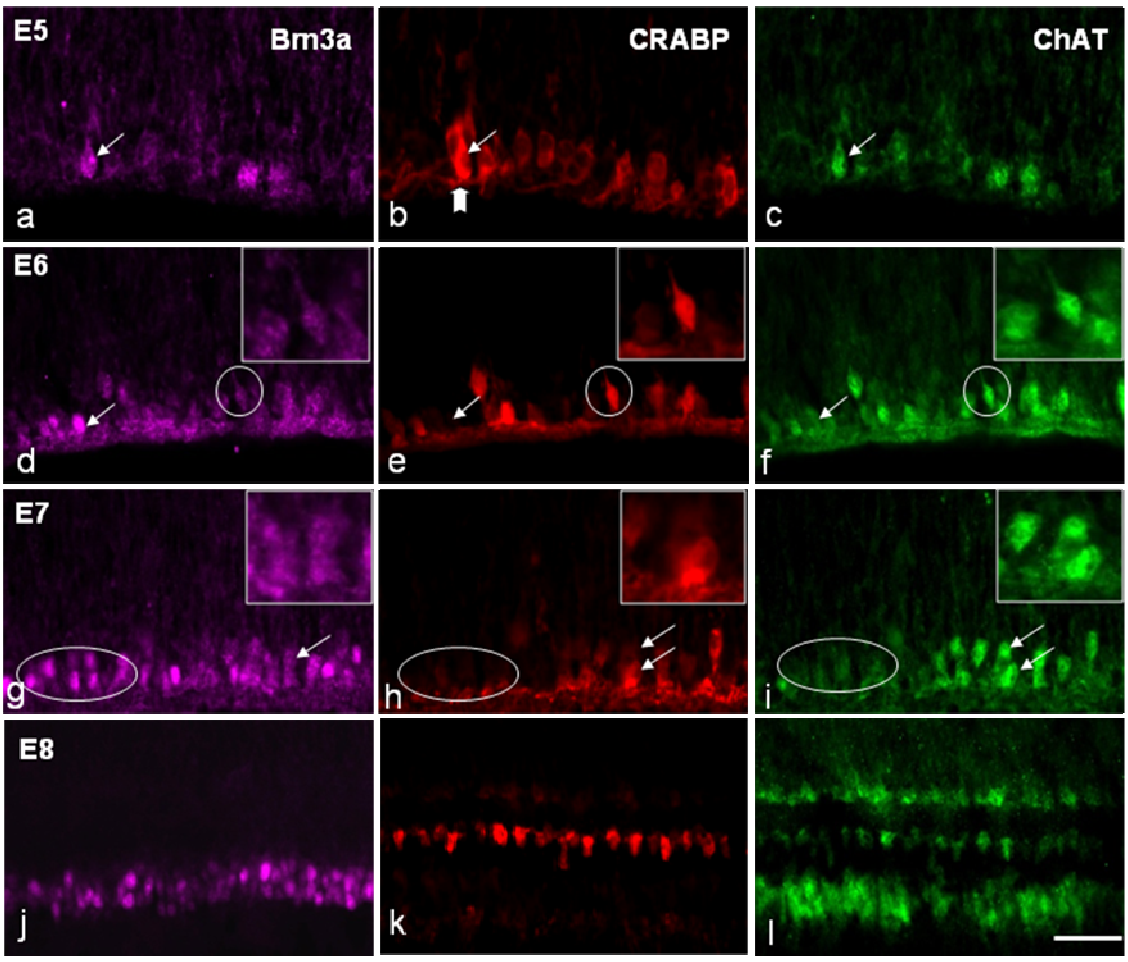

Fig. 2: E5-E8 embryonic chicken retinal sections triple stained for Brn3a (magenta), CRABP (red) and ChAT (green). Panels (a-c) show pictures from E5 retina. A few cells start to express Brn3a (a) in the ventricular side of the retina (arrow). The pictures are from the central part of the retina as the differentiation proceeds from a central to peripheral direction. Note more numbers of cells in the same region are reactive for CRABP (b). The processes from these cells are also reactive for CRABP and they extend in the cell free zone suggesting the future OFL. Note a cell which is strongly Brn3a$^+$ also co-localizes CRABP and ChAT, suggesting that the differentiating ganglion cells express ChAT (arrows in a, b and c). At E6 (d-f), more cells are reactive for Brn3a (d). These cells began to down regulate both CRABP and ChAT (arrows in d, e and f). Also notice a cell which is strongly immunoreactive for CRABP and ChAT, is mildly stained for Brn3a (shown in circles in d, e and f. Look at inserts for higher magnification). Panels (g-i) show pictures of E7 retina. More cells expressed Brn3a compared to earlier stages. All these cells are found in two or three layers forming the future GCL. The most differentiated GCs completely down regulated CRABP and ChAT (shown in ellipsoid in g, h and i. See inserts for higher magnification). Notice a group of cells a little farther which are intensely stained for CRABP and ChAT, suggesting the beginning of differentiation of cholinergic amacrine cells (arrows in g, h and i). Panels (j-l) show pictures from an E8 retina. All the Brn3a$^+$ cells have settled in the GCL (j) and none of them are immunoreactive for CRABP (k). The type II cholinergic amacrine cells occupied an intra plexiform position and expressed CRABP (k). ChAT is expressed in both type I and II SACs. Some ChAT staining is still observed in the ganglion cells. Scale bar = 50 µm.

No Brn3a reactive cells were found further inside the retinal tissue, suggesting that the differentiating GCs become specified only after having reached the vitreal side of the retina. Ganglion cells also stained for CRABP (Fig. 2b arrow) consistent with earlier reports (Mey et al., 2001). The processes emerging from the GCs which form the optic fibers also immunostained for CRABP (Fig. 2b block arrow). At E6, more cells became Brn3a$^+$, spreading from central towards periphery. Around E7, most of the GCs down-regulated CRABP but were strongly stained for Brn3a (Fig. 2g, h). By E8, all GCs reached the GCL which was clearly separated from the inner nuclear layer (INL) by a well defined inner plexiform layer (IPL). By this time none of the GCs expressed CRABP anymore (Fig. 2j, k), and the protein became restricted to the type II cholinergic amacrine cells.

Ganglion cells are transiently cholinergic during differentiation

The differentiating GCs surprisingly expressed choline acetyltransferase (ChAT) at E5 which had never been reported before. All the Brn3a$^+$ cells which appeared during this stage also co-localized with ChAT and CRABP (Fig. 2a-c). Since amacrine cells become intermingled with GCs at the vitreal side, one could misinterpret that these represent future amacrine cells which might have started to differentiate into cholinergic cells. But the expression of Brn3a, which is a specific marker for GCs, suggests that these cells in fact represent early ganglion cells which express ChAT during their differentiation. However, the expression of ChAT by GCs was transient and was observed only until E7 (Fig. 2a-i). Some staining was observed in the GCL at E8, which might be due to the release of the enzyme by type II cholinergic cells which are located in close proximity to the GCs (Fig. 2l). After maturation of the retina in an adult chick, both the cell populations were clearly observed. The GCs which were mixed among the type II cholinergic amacrine cells did not express ChAT (Fig. 1).

Early starburst amacrine cells mildly express Brn3a over a short period of time

Differentiation of a cell type might influence the expression pattern of the neighboring cell types. This mode of expression pattern was observed in our study. When the GCs began to express Brn3a, the neighboring SACs also mildly

expressed Brn3a which was transient over a very short period of time (circles in Fig. 2d, e and f). As mentioned above, the GCs expressed ChAT between E5 and E7 and gradually became intensely stained for Brn3a. The GCs increased the expression of Brn3a as a process of specification and at the same time they down-regulated the expression of ChAT and CRABP.

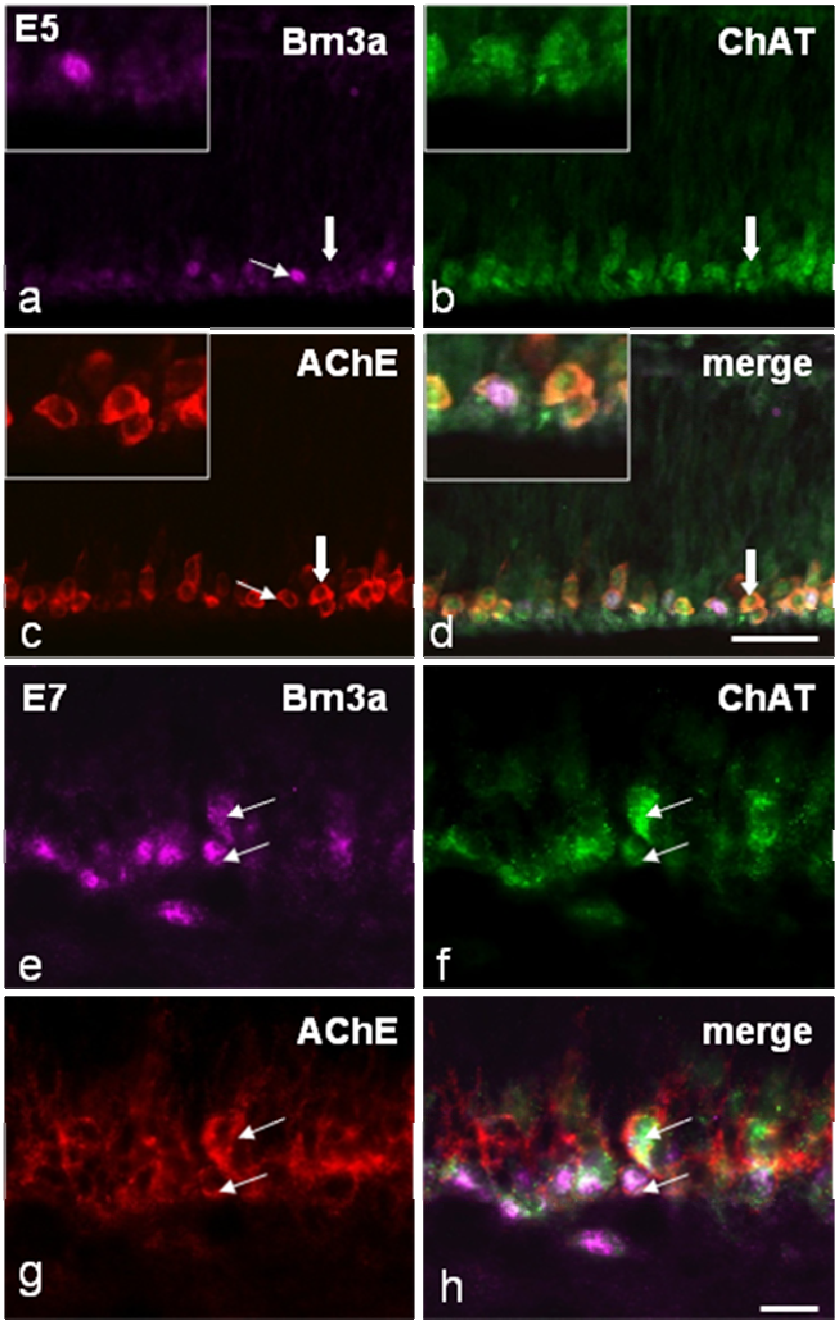

Fig. 3: Comparison of expression pattern of AChE, ChAT and Brn3a. Panels (a-d) show pictures from an E5 retina triple stained for Brn3a, ChAT and AChE. Panel (d) is the merge of all three channels. Note a Brn3a^{+} ganglion cell also contains AChE on the cell surface (arrows in a and c. The cells are shown in a closer view in the inserts). A neighboring cell which is not stained for Brn3a contains more AChE and ChAT, suggesting that these cells would become the future cholinergic amacrine cells (shown in block arrows in a-d). Scale bar = 50 µm (a-d); 20 µm (e-h).

In a similar fashion, the future cholinergic amacrine cells which are believed to be appearing around E6 (Spira et al., 1987; own observation), mildly expressed Brn3a but strongly expressed ChAT and CRABP (Fig. 2). By E8, none of the SACs expressed Brn3a any longer, while the expression of CRABP became restricted only to type II SACs.

Consistent with earlier reports, the GCs expressed AChE even before they became specified (Fig. 3a-d). These $AChE^{+}$ GCs were identified by their position and soma size at the vitreal side of the retina. These cells co-localized for Brn3a and ChAT, further confirming that GCs are transiently cholinergic during early retinogenesis.

Discussion

Retinal ganglion cells are the first cells to begin their differentiation (Kahn, 1974; Snow and Robson, 1994). Beginning their differentiation around E3/E4 (Kahn, 1973, 1974), the ganglion cells complete the fundamental differentiation processes around E13 (Nishimura, 1980). Based on these studies, it is obvious that differentiation is not a single step process immediately after the cells exit the cell cycle. The GCs are reported to begin their differentiation within 15 min after the cell cycle exit based on the expression of an early axonal protein RA4 in the migrating cells (McLoon and Barnes, 1989). Though the cells were under the differentiation phase, they still had bipolar processes connecting the cells to the ventricular and vitreal surfaces. Golgi impregnation studies showed that early events of differentiation include the shortening of bipolar processes and development of dendrites (Nishimura, 1980). Though most of the early studies which were also based on golgi studies suggested a very early stage of GC differentiation, the time of their commitment to become specified as ganglion cells is exactly not known.

The ganglion cells are closely followed by the differentiation of type I and type II starburst amacrine cells, which are the only cholinergic cells in the retina that form the basis of the OFF and ON channel dichotomy (Layer et al., 1997). These cells are derived from a common pool of migrating postmitotic cells and are identified in two rows in the presumptive IPL (Prada et al., 1999). The type II SACs are distributed in close proximity to the GCs in the developing GCL. During early periods of development, type II SACs express genes like Pax6 and Islet1 in common to the ganglion cells. In addition the type II SACs expresses neurofilaments transiently, which is a ganglion cell specific protein (Stanke et al., 2008). These starburst amacrine cells could be differentiated from the closely abiding GCs by the presence of cholinergic markers like ChAT and VAChT (Loureiro-dos-santos et al., 2002). Earlier studies reported the onset of ChAT expression in these cells to be around E6 (Spira et al., 1987). The environmental signal which triggers the onset of expression of ChAT in the amacrine cells that specifies them to become the future cholinergic cells remains unknown.

We employed specific immunohistochemical techniques to identify differentiating and committed ganglion cells based on the expression of POU-domain

transcription factor, Brn3a. Additionally ChAT, CRABP and AChE were used as markers to identify the differentiating SACs. Based on our observation, we report here that differentiating GCs transiently express CRABP and ChAT during early embryonic stages (E5/E6), while AChE expression in these cells continues throughout the adult retina.

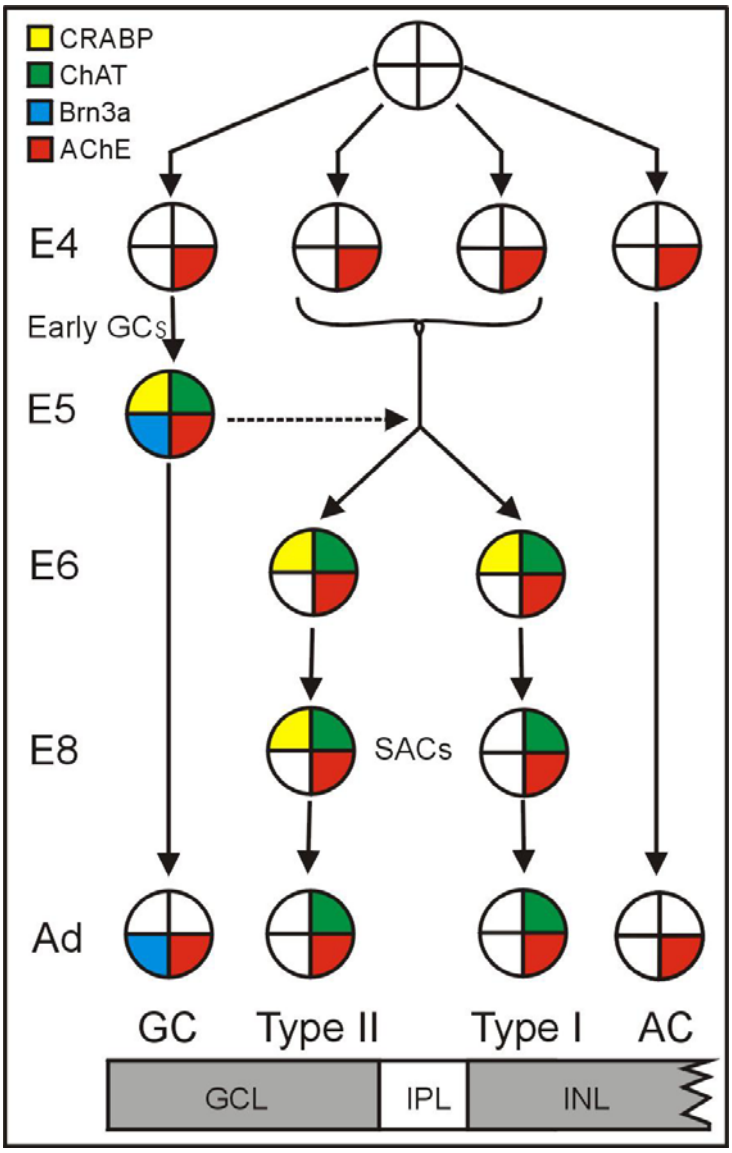

Fig. 4: Schematic representation of the possible way of differentiation and specification of ganglion and starburst amacrine cells. At E4, the cells in the central part of the retina are $AChE^{+}$, during which the GCs might have already begun their differentiation but still not specified. GCs expressed Brn3a (blue) for the first time at E5 but also co-localized for CRABP (yellow), ChAT (green) and AChE (red). After E6/E7, the GCs retained Brn3a and AChE but had lost ChAT and CRABP, thus marking the specification of ganglion cells. On the other hand, SACs began to accumulate ChAT and CRABP along with AChE during differentiation. By E8, the type I SACs became specified by loosing CRABP, while the type II SACs retained the protein. In an adult stage, the type II cells had also down regulated CRABP. The last column indicates the other population of amacrine cells which express only AChE and occupy the INL.

The transient expression of CRABP and ChAT in the differentiating GCs might suggest early morphogenetic functions of retinoic acid and acetylcholine.

The transient expression of CRABP and ChAT in the differentiating GCs might suggest early morphogenetic functions of retinoic acid and acetylcholine. Co-localization of the cholinergic marker ChAT, CRABP and Brn3a at embryonic stages E5/E6 suggest that the ganglion cells are still not committed, although differentiating. At E7/E8, all the $Brn3a^{+}$ cells had no more ChAT and CRABP expression. Though the expression of CRABP in the GCs was already reported by Mey et al., 2001, ChAT expression in these cells was never observed before.

The beginning of Brn3a expression is observed only at E5 and becomes stronger by E6/E7 as the GCs become more specified. Interestingly, a mild expression of the transcription factor could be observed in the future cholinergic SACs which have started to accumulate CRABP and ChAT. This mild expression could be due to the local contact mediated signaling that might induce a transient gene expression in common to the neighboring cells (Lucia Galli-Resta., 2000).

The expression of ChAT is never observed before E5/E6 in the migrating amacrine cells. At the same time the future cholinergic type I and type II cells which arise from a common pool of postmitotic cells are already arranged in two rows in the presumptive IPL, there should be some cellular signals that trigger these cells to express ChAT. As we observe ChAT expression as early as E5 in the GCs, we propose that these early GCs might provide the necessary signals to the future cholinergic cells to express ChAT. Based on these observations we proposed a possible scheme of cells being committed either as ganglion cells or future cholinergic amacrine cells (Fig. 4). However, the reason for transient expression of CRABP in the ganglion cells around E5/E6 and then in the type II SACs around E7/E8 till E16 (Stanke et al., 2008) is not yet understood.

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Chapter III

This chapter has been published as Simple explant culture of the embryonic chicken retina with long-term preservation of photoreceptors (2011). Exp Eye Res 93(4) 556-564.

Appropriate *in vitro*-model systems are instrumental in order to follow the development of retina from early stages and also to compare the differentiation patterns of SACs and inner plexiform layer. In this chapter, a model of an in vitro retinal explants system which closely mimics in vivo retina and its advantages is described.

Simple explant culture of the embryonic chicken retina with long-term preservation of photoreceptors

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Keywords: Chick retina; explant; rotation culture; cytoarchitecture; development; long term; photoreceptors; outer segments.

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Abstract

Structurally stable in vitro-model systems are indispensible to analyse neural development during embryogenesis, follow cellular differentiation and evaluate neurotoxicological or growth factor effects. Here we describe a three-dimensional, long-term *in vitro*-culture system of the embryonic chick retina which supports photoreceptor development. Retinal tissue was isolated from E6 chick eye, and cultured as explants by continuous orbital rotation to allow free floatation without any supporting materials. Young stage (E6) immature retinas were cultured for various time periods in order to follow the differentiation of cell types and plexiform layers by immunocytochemical methods. These explants could be cultured for at least 2-3 weeks with remarkable retention of retinal architecture. Interestingly, photoreceptors developed in the absence of pigment epithelium. Electron microscopic studies revealed formation of structures resembling photoreceptor outer segments, a feature not reported previously. Thus, the verification of photoreceptors, Müller cells, inner retinal cells and the inner plexiform layer described in our study establishes this explant culture as a valuable *in vivo*-like model system.

Introduction

The need for well-organized *in vitro* culture systems, which can mimic an *in vivo* situation, is still a high priority, in spite of remarkable advances made since the last century. The central nervous system (CNS), especially brain or retinal tissues from rat (LaVail and Hild, 1970; Rzeczinski et al., 2006), mouse (Caffé et al., 1989), and chicken (Kato et al., 1982; Hoff et al., 1999) were highly exploited in the development of *in vivo*-like culture systems that could be employed in different areas of study including embryonic development, cellular differentiation, pharmacology, transplantation (Johnson and Martin, 2008; Johnson et al., 2009) and other medical aspects.

Several methods have been developed for culturing retinal explants from different species. Earlier techniques involved the usage of different substrata on which retinal tissues were placed for support, including plasma clots (Barr-Nea and Barishak, 1970), and chicken gizzard extracts (Kato et al., 1982). Histotypic differentiation of mouse retina was obtained when tissues were explanted flat on a piece of nitrocellulose membrane (Caffé et al., 1989). Successful preservation of the cytoarchitecture over a period of several weeks was observed in slice culture techniques from rodent CNS (Gähwiler et al., 1997). Organotypic organization in chick retinal explants was demonstrated when the tissues were embedded in a fibrin clot on glass cover slips and rotated in roller tubes (Hoff et al., 1999). Recent developments have paved ways to culture retinal slices from adult rat retina by free floating them in roller drums under constant rotation (Rzeczinski et al., 2006).

Though complete retino-architecture was obtained and maintained in retinal explants for long culture periods, in either standard or roller cultures, no system has been established up to now that could preserve the development of photoreceptor outer segments. Studies show that photoreceptor outer segments developed to a certain extent when explants were cultured in the presence of retinal pigment epithelium but totally failed in the absence of the latter (Caffé et al., 1989). Electron microscopic studies also suggested the absence of photoreceptor outer segments in chick retinal explants (Araki et al., 1987).

Our aim was to establish an *in vivo*-like explant culture system from chick that differed significantly from other systems developed so far. The main difference compared to other explant systems was the absence of any substratum used as a support for the tissues. Instead of slicing the retinal tissues, we used whole retinal tissues as explants which were subjected to continuous and constant orbital rotation. The explants were cultured in a spacious dish rather than using narrow roller tubes, in which they were allowed to float freely in order to obtain a 3D culture environment. We report here a simple but effective culture technique that allowed us to maintain the retinal architecture for at least 2-3 weeks. During the culture periods, the explants reached a near *in vivo*-like tissue organization. Since we used embryonic chick retina, we were able to follow complete differentiation of all retinal layers, starting from an early neuroblastic stage until the development of photoreceptor outer segments.

Materials and Methods

Fertilized eggs of White leghorn chicken were purchased from a local hatchery and incubated at 37°C in a humidified chamber until 6, 10 and 15 days of embryonic development for explant cultures. Different stages starting from embryonic day 6 – 17 were used to perform parallel *in vivo* retinal development studies. For explant cultures, eggs were opened at embryonic day 6 (E6) and the eyes collected in calcium free Hank´s Basal Salt Solution (HBSS). Using a fine scissors the lens was removed by dissection along the ora serrata in such a way that most of the peripheral retina was lost. The vitreous body was next removed with sharp forceps. The central retina which was associated with the pigment epithelium was removed carefully with a spatula without any contamination of the latter. The whole retinal cup was flattened with the ganglion cell layer facing upwards without any damage and cut into two equal parts with a micro scissors (see Fig.1). For *in vivo* studies, whole eyes of different stages were collected.

Establishment of explant culture

The equal retinal halves were transferred to 35 mm culture dishes containing DMEM (Dulbecco's Modified Eagle's Media) with a supplement of 10% fetal calf serum, 2% chicken serum, 1% L-glutamine, and 0.15% penicillin/streptomycin (all from Gibco, Berlin, Germany). No substrata such as Millipore filters or poly-cationic substances were used in order to plant the retinal tissues, but, instead, the dishes with explants were placed on a self-made rotatory shaker installed in a Heraeus incubator and allowed to rotate continuously in an orbital manner with a constant speed of 72 rpm. The explant culture was supplied with 95% air, 5% CO_2 and maintained at 37°C throughout a culture period of 2-3 weeks. Fresh medium was changed every second day. Due to continuous rotation and avoidance of substrata, the tissues were not allowed to adhere to any surface, and thereby kept free-floating and preserving their 3D architecture.

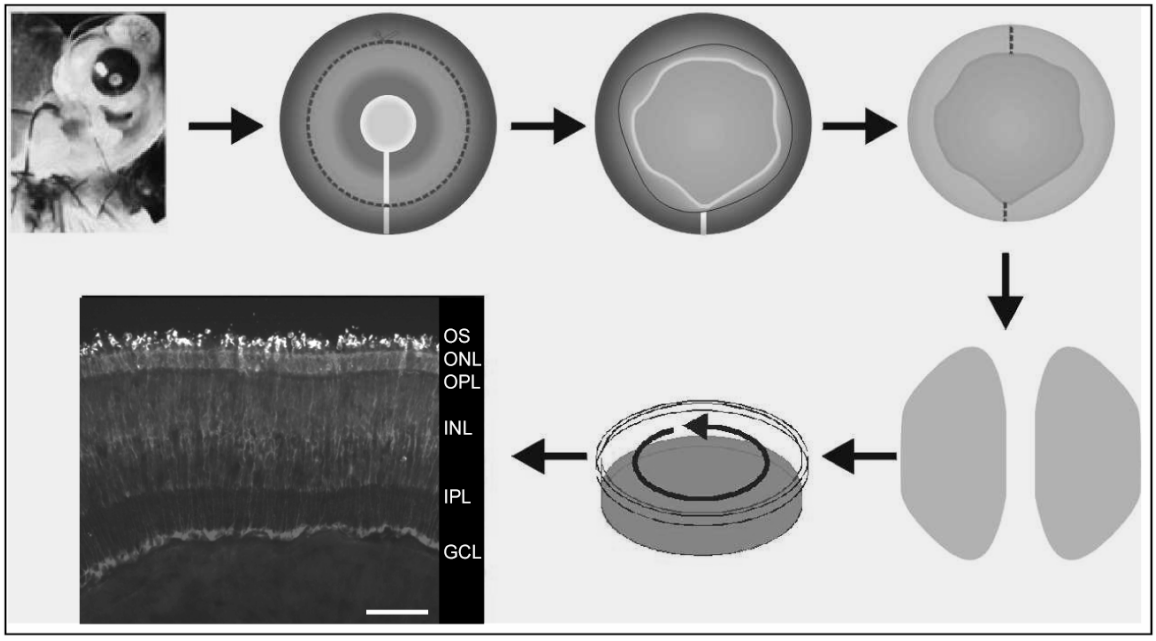

Fig. 1: Schematic representation of the method of preparing explants from an E6 chick embryo. Eyes from E6 embryos were removed and whole retinal cups were isolated without pigment epithelium. The tissues were cut into two equal parts and seeded in 35 mm dishes with medium in the absence of substratum. Constant orbital rotation (72 rpm) results in the growth of retinal explants in a 3D-like environment.

Fixation and cryosectioning

The explants from different culture days beginning from div 2 till 20 were collected in Eppendorf tubes with 1 ml tips which were cut at the tip to have a wide mouth. The tissues were washed thrice with PBS (Phosphate Buffered Saline) and fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) for an hour and immersed in 25% sucrose (Roth, Karlsruhe, Germany) overnight at 4°C.The fixed explants were transferred to tissue-tec (Richard-Allan Scientific) solution and sectioned on a cryostat (Microm, Heidelberg, Germany) at 10-12 µm and mounted on frost-free or gelatine-coated glass slides. The sections were stored at -20°C until further use. Whole eyes for *in vivo* studies were also fixed in 4% paraformaldehyde overnight at 4°C, immersed in 25% sucrose and sectioned as whole mount eyes as mentioned above.

Immunocytochemistry

Sections of explants and in vivo whole mount eyes were subjected to immunostaining in order to follow and compare the development and differentiation of different types of retinal cells in both the systems. Prior to staining procedures,

the frozen sections were dried on a heating plate at 37°C. The area around the sections was marked with a greasy liquid blocker (Roth, Karlstuhe, Germany) and the slides were pre-incubated in blocking solution which contains PBS, 3% BSA (Bovine Serum Albumin) and 0.1% Triton-X-100 for at least 30 min. 100 µl of primary antibodies which were diluted in the blocking solution were applied on the sections inside the area which was surrounded by the liquid blocker and then incubated for 90 min at room temperature or overnight at 4°C. In case of double staining, one polyclonal and one monoclonal antibody was mixed and applied together. The slides were washed thrice with PBS and then subjected to 100 µl of fluorescent conjugated secondary antibodies for 70 min at room temperature. After three PBS washes, the sections were treated with DAPI (Merck, Darmstadt, Germany) for 3 min in order to stain cell nuclei. The slides were rinsed in distilled water after a final wash in PBS and then dried on the heating plate and embedded in Kaiser's glycerol gelatine (Merck, Darmstadt, Germany). The details of primary and secondary antibodies, their dilutions and sources used in this study were as follows: 3D10 antibody binding to AChE, anti-Pax6 for inner retinal cells, H5 (vimentin) and glutamine synthetase antibodies specific for Müller glial cells, and 4H6 antibody specific for neurofilaments were all monoclonal. 3D10 and glutamine synthetase antibodies were used at a dilution of 1:200, and vimentin antibody was used at a dilution of 1:500. Anti Pax6 was used in a dilution of 1:500 and 4H6 at a dilution of 1:250. Polyclonal antibodies CERN 901 and CERN 906 were used at a dilution of 1:1500 to recognise rod and cone photoreceptors. Fluorescent-coupled secondary antibody Cy3 donkey-anti-mouse was used against monoclonal antibodies, and Cy2 goat-anti-rabbit was used against polyclonal primary antibodies. Both the secondary antibodies were used at a dilution of 1:200. Sources of antibodies: 3D10 antibody was a generous gift from Dr.Karl Tsim, Hongkong, H5, 4H6 and anti-Pax6 were purchased from Developmental Studies Hybridoma Bank, glutamine synthetase from BD Biosciences, CERN 901 and 906 antibodies were generous gifts from Dr.William J. DeGrip, Netherlands and the secondary antibodies Cy2 and Cy3 were from Dianova.

Cholinesterase staining

Acetylcholinesterase staining was performed by the Karnovsky and Roots technique (Karnovsky and Roots., 1964). In brief, the frozen sections were dried on a heating plate, incubated twice for 15 min in Tris-maleate buffer and treated with the reaction buffer (0.1M tris-maleate buffer, 0.1M sodium citrate, 30mM copper sulphate and 5mM potassium hexacyanoferrate).The reaction was performed for 1 hr at 37°C with 6 mM acetylthiocholine iodide as substrate and 0.1 mM iso-OMPA to inhibit BChE. The reaction produced a brown insoluble precipitate (Hatchett´s brown) at the site of enzymatic activity which was visible under the light microscope.

Microscopy and Imaging

Immunostained *in vivo* and explant sections were subjected to fluorescent microscopy (Axiophot, Carl Zeiss, Germany) which was connected to an automated digital camera (Axiocam). Imaging was done for each fluorescent channel separately and processed through extended focus imager, and all the pictures from different channels were combined as overlays and stored in zvi image format by Axiovision Basis software programme.

Electron microscopy

Eyes from E17 chick embryos and div 17 retinal explants were sectioned, washed three times with PBS, and fixed in a mixture of 2.5% glutaraldehyde and 1% formaldehyde in cacodylate buffer, pH 6.8 for 1hr. The sections were then post-fixed in 2% osmium in cacodylate buffer for 2hr at room temperature. The sections were then contrasted with 2% uranylacetate at 4°C overnight. After contrasting, the tissues were dehydrated in a graded series of acetone (30 – 100%) and finally embedded in SPURR (3, 4 Epoxycyclohexylmethylcarboxylate, D.E.R 736, Nonenyl-Bernsteinacid and Dimethylaminomethanol) for polymerisation at 74°C for 24h. Ultrathin sections were cut, stained with uranylcitrate and viewed under a Zeiss electron microscope.

Statistical studies for the efficiency of explants to form organized structures from different embryonic stages

At least 24 explants of different embryonic stages (E6, E10 and E15) from each corresponding culture day were used in order to calculate the efficiency of explants in preserving organized structures. All the explants were cryosectioned and stained for DAPI in order to view the formation of different cellular layers. The total number of explants from each embryonic stage that maintained the structures was scored as percentage of organization against the number of culture days. The efficiency of explants to produce and maintain organized structures are represented stastistically.

Analysis was done using Graphpad Prism software. Statistical significance was assessed by one-way ANOVA (Analysis Of Variance) followed by Tukey tests for multiple groups. Differences among embryonic stages were considered significant for $p<0.05$.

Results

A simplified culture system

We implemented a simple but effective technique that allowed us to culture the explants not only for long periods but also to give us an opportunity to culture them without the need of any external substratum. The simple strategy of rotating the explants in an orbital direction resulted in folding of the retinal tissues (see Fig. 2, a). This folding of explants might occur due to massive proliferation and the differential polarity of the different neuronal cell types. The formation of rosettes was rare within the explants and occurred at places where the photoreceptors came into close proximity due to folding of the explants (Barr-Nea & Barishak, 1970), but most of the explants were devoid of rosettes. Since the whole retinal cup was divided only into two equal parts, most of the tissue injuries which might arise due to slicing were avoided. Embryonic day 6 (E6) retina was routinely used because i) at this stage the retina is not fully mature which allowed us to follow retinal development, study the differentiation processes of cells and compare it with *in vivo* development ii) the pigment epithelium is not completely developed at this stage which makes the isolation procedure simple.

Preservation of cytoarchitecture

The retinal cytoarchitecture was completely preserved in the explants for nearly 2-3 weeks of culture (see Fig. 2, a and b). Since no substratum was used in our culture system, the explants were completely independent from any supporting materials during their growth. In contrast to monolayer cultures, where Müller cells adopt different morphologies and gene expression patterns, our culture system retained a structure that much more closely resembled normal *in vivo*-behavior, which is vital for preservation of the retinal tissue architecture. A rotation speed of 72 rpm provided an ideal environment for the explants. A higher rotation speed resulted in tissue damage due to excessive frictions and breakage, while very low rotation speed resulted in adherence of tissues either to their neighbours, or the samples tended to settle to the bottom of the culture dishes (data not shown). Importantly, at optimal rotation speed the photoreceptor outer segments could develop and were not lost by friction. However, there was thin-

ning of the ganglion cell layer (GCL) due to the loss of ganglion cells which is a normal consequence of severing the optic nerve.

In addition to E6 retinae, E10 and E15 retinal tissues were used as explants to compare the efficiency of explants in forming regular and higher order organotypic structures. Explants from E6 and E10 were able to survive the culture conditions even until 3 weeks while E15 explants did not keep their structures after one week (see Fig. 3).

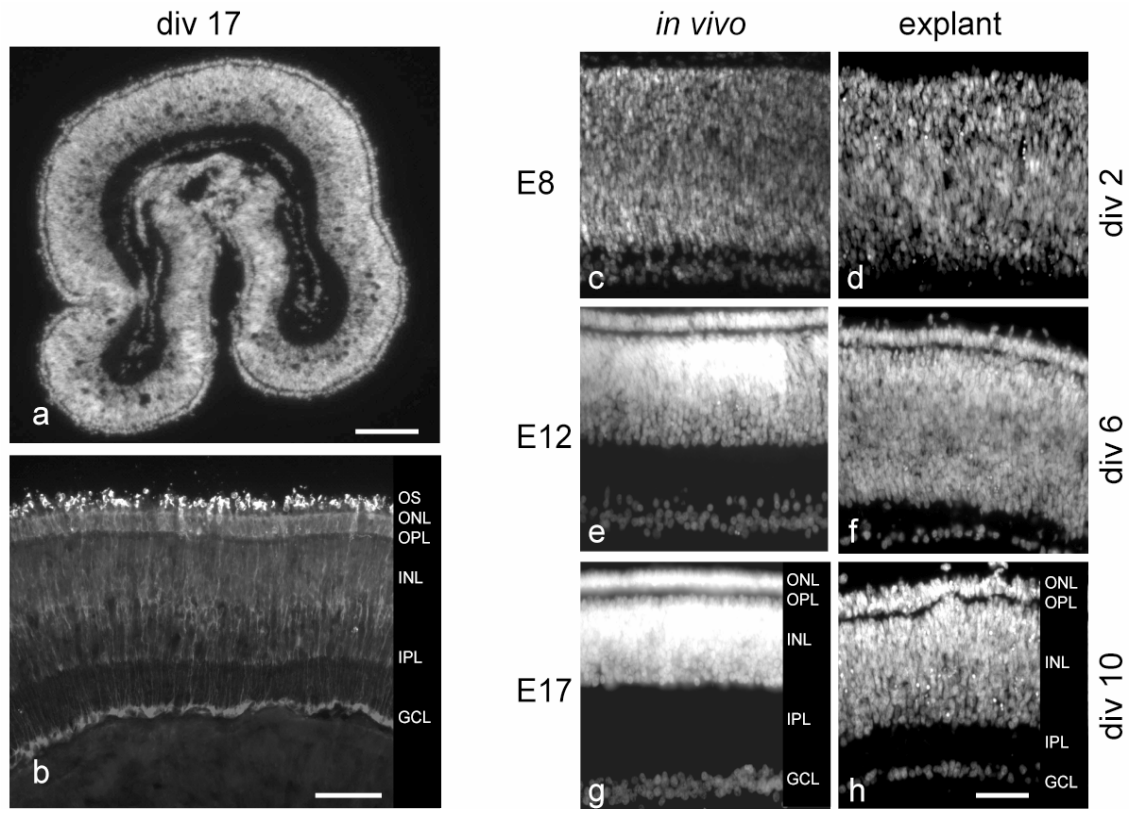

Fig. 2: Preservation of retinal architecture. (a) DAPI-stained whole explant is shown after 17 days in culture. Note the folding and tendency of explant towards fusion. (b) An explant cultured for 17 days and immunostained for CERN 901(rod photoreceptors) and glutamine synthetase (Müller cells) showing the preservation of its cytoarchitecture. Comparison of the formation of retinal structures and plexiform layers in explants and *in vivo* by DAPI staining is shown. Three different stages of *in vivo* retina (E6, E12 and E17) are shown in the left lane (c, e and g). Three different stages of explants (day 2, 6 and 10 *in vitro*) are shown in the right lane (d, f and h). Note the similarities in formation of different layers in both cases. Scale bar, 50 µm (a, c, d, e, f, g and h); 20 µm (b).

Organotypic differentiation: a model comparable to in vivo development

DAPI staining of the cryosectioned explants revealed a high order of organotypic differentiation in the explants which provided an excellent model system highly comparable to the *in vivo* retinal development (see Fig. 2 c-h). After 2 days of explant culture, the cells appeared similar to neuroblasts of E8 *in vivo* ret-

inas. After 3 div, an IPL was first discernible (data not shown), and on the 4th day of culture, the separation of OPL and ONL from the INL could already be noticed. By 8 div, the explants almost appeared like a mature retina with proper formation of the nuclear and plexiform layers. A few pyknotic cells were seen throughout the tissues which might have been the result of apoptosis.

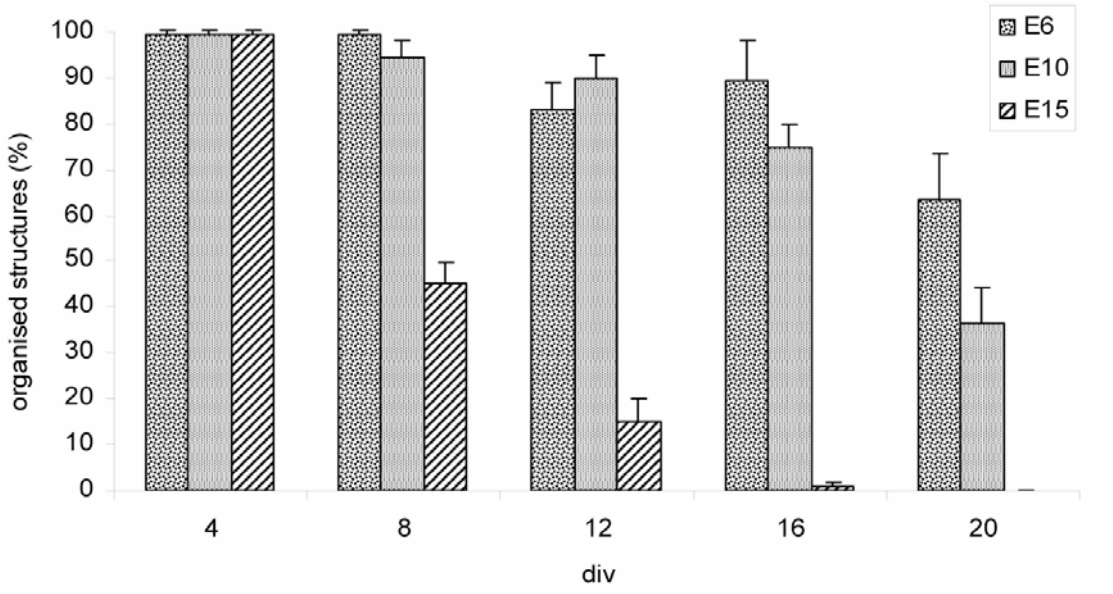

Fig. 3: Efficiency of explants to produce organized structures from retinas of different embryonic stages. Statistical data representing the efficiency of explants from different embryonic stages was plotted comparing the percentage of organized structures against the number of days in culture. In addition to E6 retinal tissues, retinas from E10 and 15 were used for explant culturing. Explants were cultured for various periods as indicated. Organized structures were revealed by DAPI staining (refer to Fig.2a). Graph represents data from three independent experiments with *n*=24 for each embryonic stage. Statistics was performed by one-way ANOVA. Data are represented as means (n=24) +SE, (**p<0.01, ***p<0.001).

Formation of an IPL

In the inner plexiform layer, neuropil from different types of amacrine cells, ganglion cells, and also bipolar cells become interconnected within highly stratified synaptic sublaminae. Specifically, amacrine cell types organize their processes within two main sublayers between cells of the inner nuclear layer (INL) and the ganglion cell layer (GCL). Acetylcholinesterase (AChE) is an early marker during the formation of the IPL, and AChE immunohistochemistry was used here to demonstrate the IPL development in explants. AChE was clearly detectable in E6 embryos, the earliest examined time point (data not shown). At 2 days of culture, AChE was found in the developing IPL as a narrow faint band which

paralleled the stage of E8 *in vivo*. By 4 days *in vitro*, the AChE band had become broader, resulting in further separation of the amacrine cells to the GCL (data not shown). By 10 days *in vitro* onwards, AChE was detectable in two major subbands in the IPL, exactly paralleling the stages of *in vivo* (see Fig. 4). A faint AChE staining was seen in a population of cells at the outer border of the INL, corresponding to the population of horizontal cells. AChE enzyme activity by Karnovsky-Roots staining also revealed two IPL subbands in explants, and a weak staining in the horizontal cell population. A thin layer of stained cells found in the GCL were displaced amacrine cells. The IPL was relatively small compared to *in vivo* retina and might be due to the absence of ganglion cells. Staining with anti-Pax6 antibody documented the formation of the inner nuclear layer. Though Pax6 is expressed very early during retinogenesis, it is retained by subsets of amacrine cells even at late stages of development.

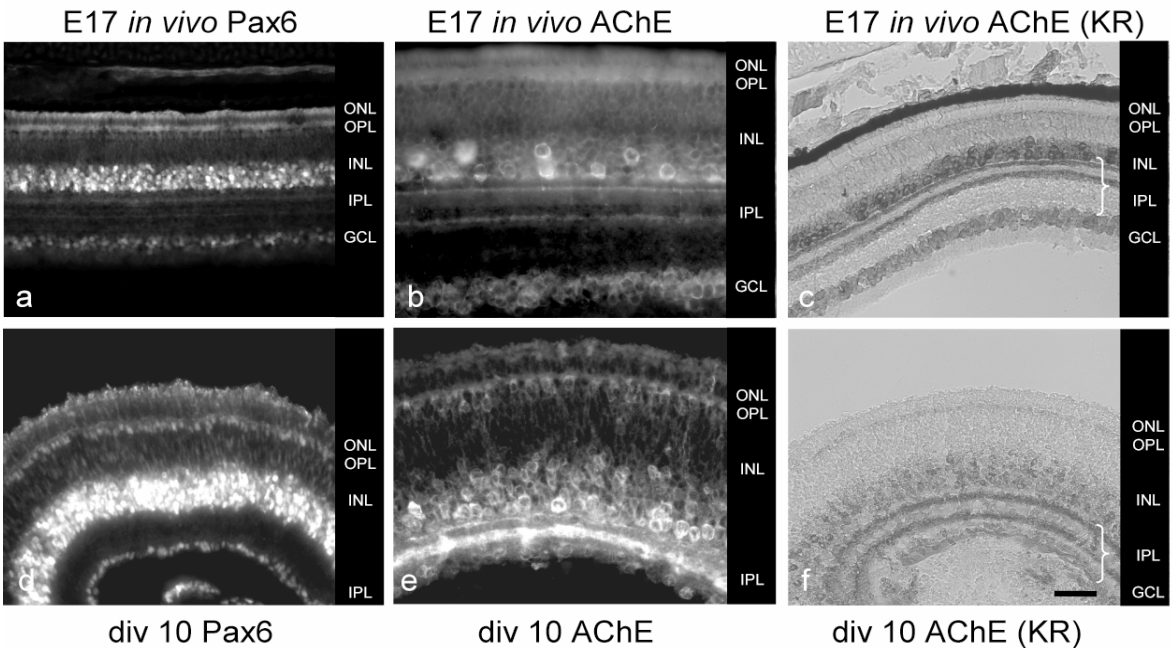

Fig. 4: Development of inner retina. Sections of *in vivo* retina and explants stained by Pax6 antibody to show the development of an INL. Pictures (a) and (d) show the inner retinal cells in E17 retina and div 10 explants, respectively. Note the similarities in the process of development. IPL development is shown by immunostaining with 3D10 antibodies against AChE and activity of the same in the IPL by Karnovsky-Roots technique (KR). (b) and (e) show the development of the IPL both *in vivo* and explants. (c) and (f) shows the development of the IPL subbands in both cases. Note the formation of IPL in explants in comparison to the *in vivo* retina. Scale bar, 50 µm (a - f).

Differentiation of Müller cells

The differentiation of Müller cells supposedly takes place late during the embryonic retinal development in vivo. This differentiation is associated with

proper organization of the retinal structure, and also with the development of photoreceptors and their outer segment formation. Müller cell progenitors express the intermediate filament protein vimentin. During their differentiation, they begin to express the enzyme glutamine synthetase, which is actively involved in transamination of extracellular glutamate to glutamine. Hence, we used both vimentin and glutamine synthetase as radial glial markers in explants.

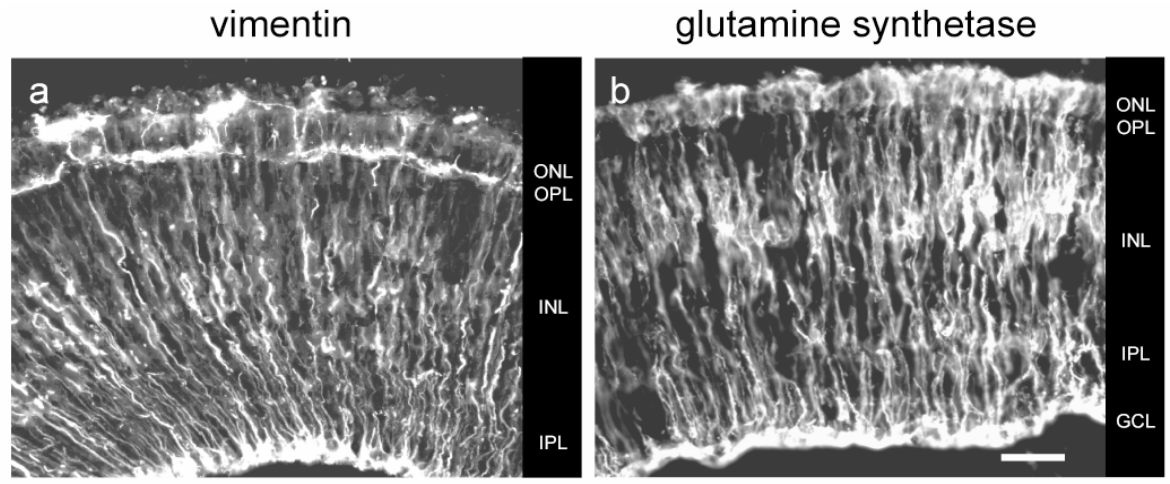

Fig. 5: Differentiation of Müller glial cells in explants. Two monoclonal antibodies, one against vimentin and the other against glutamine synthetase were used to stain div 10 explants. Picture (a) shows the radial organization of Müller cells stained for vimentin. Strong staining of glutamine synthetase of Müller cells (b) showing its presence throughout the entire retina. Dense layer of staining in the INL corresponds to the cell bodies of Müller cells. Scale bar, 20 µm (a and b).

Vimentin was found to be expressed as early as day 2 in culture (data not shown). Labelled cells were found in highly organized manner extending from the ILM to the OPL, where they were found to make horizontal contacts. Differentiation of glial cells was advanced in explants, as compared with the *in vivo* retina. Glutamine synthetase which is considered to be a differentiation marker was found to be already expressed by 6 div, a time point corresponding to 12 days *in vivo* (data not shown). By 10 div, Müller cells had spread radially throughout the explants, reaching from photoreceptors to the GCL. Their cell bodies were densely stained for glutamine synthetase and were situated in the INL (see Fig. 5).

Expression of neurofilaments in explants

Neurofilaments are intermediate filament proteins expressed by ganglion cells when they begin to differentiate. *In vivo*, neurofilament expression was seen

distributed also in the IPL as subbands with dense staining of the inner limiting membrane and the optic nerve fibers formed by the processes of the ganglion cells. Surprisingly, altough the explant cultures exhibit a “GCL” devoid of ganglion cells, neurofilament expression was still found. Two weakly stained bands were seen in the IPL when compared to five bands *in vivo*. At 4 days of culture, neurofilament expression was strong in the GCL, but no bands were seen in the IPL. At 10 div, two weak bands were seen in the IPL. This expression pattern raised the question as to which cell types expressed neurofilaments *in vitro* in the absence of ganglion cells. Though earlier studies had shown that type II cholinergic amacrine cells expressed neurofilaments for short time during their differentiation, we observed a long-term expression of these proteins in our culture system (see Fig. 6).

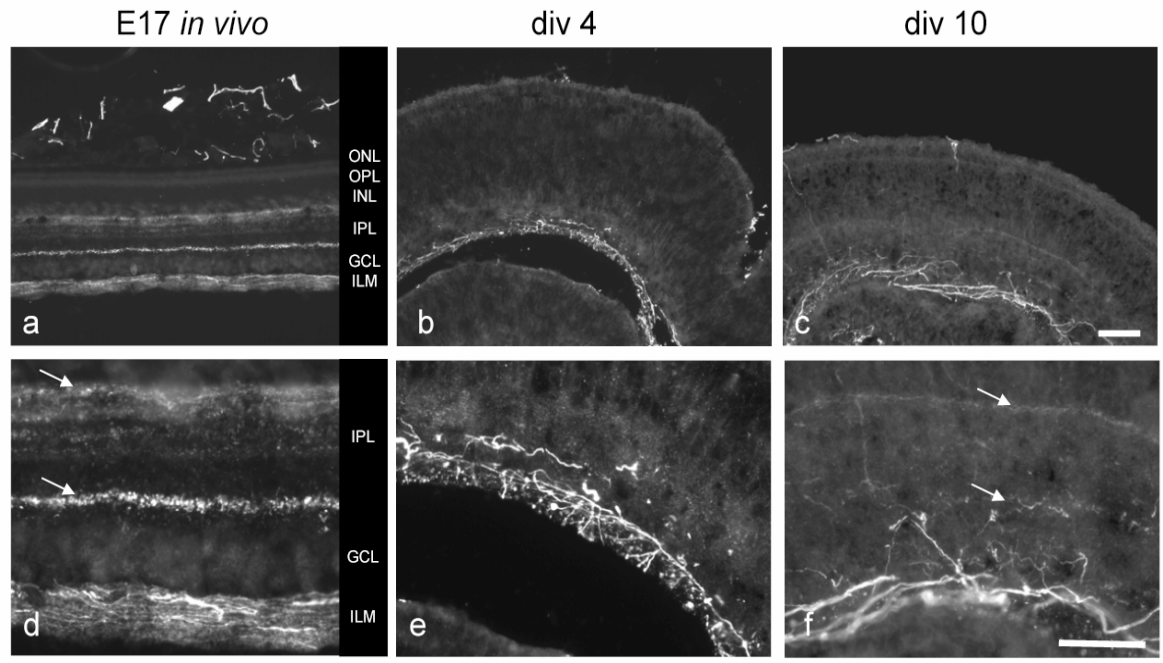

Fig. 6: Expression of neurofilaments in explants. A monoclonal antibody 4H6 specific for neurofilaments was used. Pictures (a) and (d) show the expression of neurofilaments in the IPL and ILM of E17 *in vivo* retina. Note the distribution of neurofilaments as several bands shown in between the arrows. Pictures (b and c) show the expression of neurofilaments in div 4 and 10 explants, respectively. Pictures (e and f) are higher versions of (b and c). Note the expression of neurofilaments in two thin bands in the IPL (arrows) and thick processes in the GCL. Vertical processes are seen in the IPL. Scale bar, 50 µm (a, b and c); 20 µm (d, e and f).

Photoreceptor development is advanced in explants

Though photoreceptors are born relatively early, their differentiation *in vivo* occurs only after E15. Around embryonic day 16, the photoreceptor cell bodies were oval or round and form the base of a dense stalk which leads to the forma-

tion of the outer segment. After 17 days *in vivo*, the cell bodies were found elongated, the stalk thinned and outer segments developed thereafter (data not shown). We used CERN 901 and CERN 906 to detect differentiating rod and cone photoreceptors, respectively.

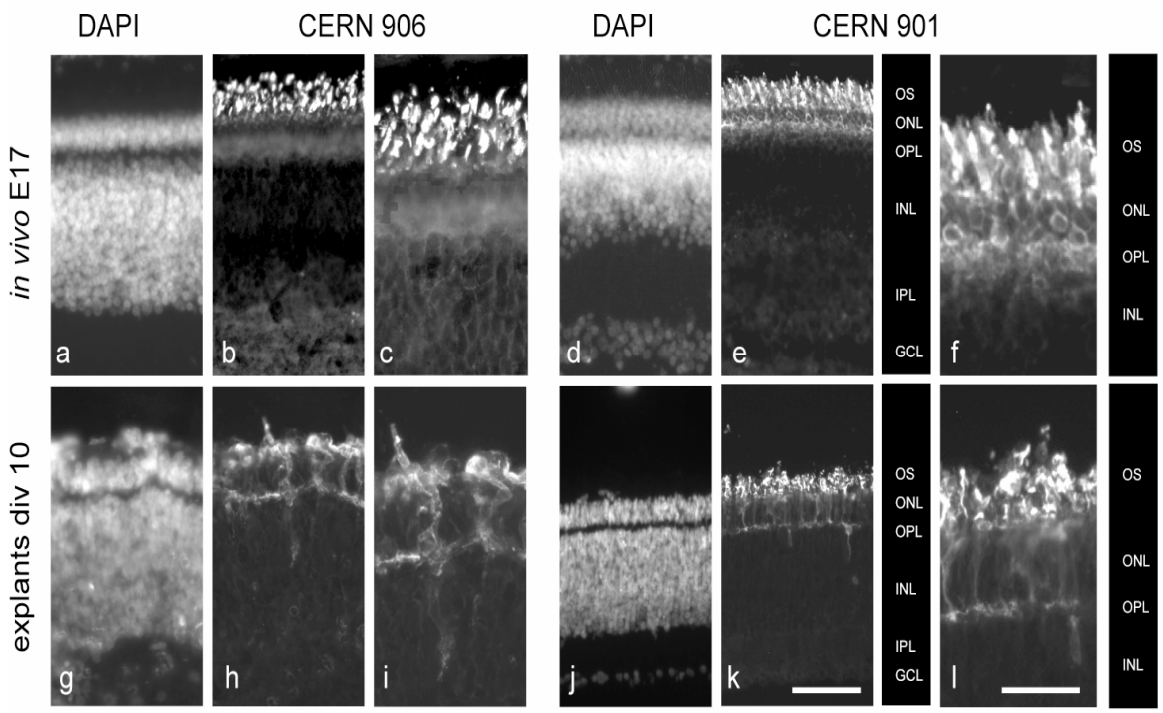

Fig. 7: Differentiation of photoreceptors in explants. The polyclonal antibodies CERN 901 and CERN 906 were used to detect the differentiation of rod and cone specific photoreceptors in both *in vivo* and in explants. Pictures (a, d, g and h) showing the structure of retina and its layers by DAPI staining in *in vivo* (E17) and explants (day 10), respectively. Pictures (b, c, e and f) show the differentiation of photoreceptors in vivo while pictures (h, i, k and l) show the photoreceptor development in explants. Note the development of photoreceptor outer segments at higher magnification (c, f, i and l). Scale bar, 50 µm (a, b, d, e, g, h, j and k); 20 µm (c, f, i and l).

At the onset of differentiation, the protein was found to be expressed strongly in cell bodies. During late stages of differentiation, when the outer segments were formed, the staining became restricted to the outer segments although the cell bodies were also weakly stained. In explant cultures, we could follow the same differentiation pattern except for the time sequence. Explants showed an advanced photoreceptor differentiation. Stainings by antibodies CERN 901 and CERN 906 demonstrated the development of rhodopsins and cone opsins as early as 4 days of culture (data not shown), which is equivalent to 10 days *in vivo*. By 10 div, differentiation of photoreceptors and formation of outer segments became clearly visible (see Fig. 7). This advanced development might

have been due to known and unknown factors present in the serum. Moreover, this early maturation *in vitro* provides an advantage to study the development of the entire retinal structure before the explants reached their culture endpoints.

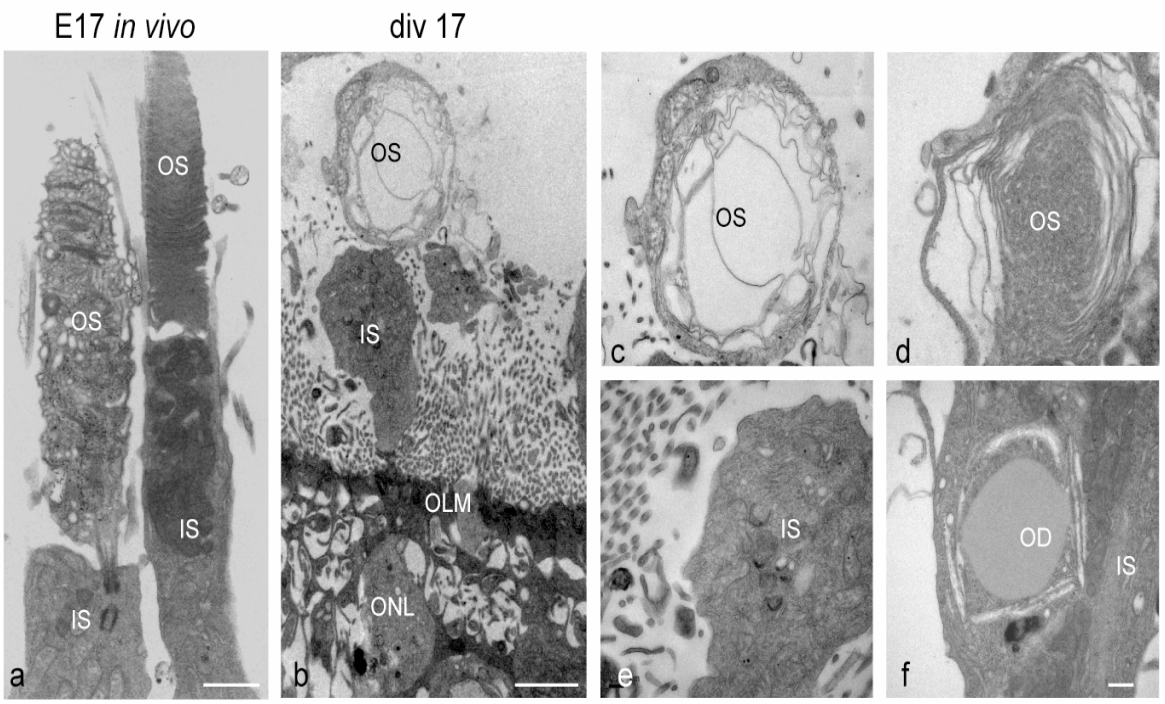

Fig. 8: Electron microscopic studies on photoreceptor outer segments. Picture (a) shows high resolution electron micrograph of inner and outer segments of photoreceptors from E17 *in vivo* retina. Picture (b) shows a photoreceptor cell in the ONL with the inner segment and structure resembling outer segment in explants cultured for 17 days *in vitro.* Pictures (c and e) are higher magnifications of inner and outer segments. Notice the elaboration of outer segments, presenting stacked, but diffusely arranged membranes (c). Pictures (d and f) are outer and inner segments from different explants. Notice closely arranged membrane stacks in the structures resembling outer segments (d). Also note an oil droplet formed in the inner segment of a photoreceptor (f). Scale bar 1 µm (a); 2 µm (b); 0.5 µm (c, d, e and f).

In order to confirm that the explants produced photoreceptor outer segments, electron microscopic studies were performed on ultra-thin sections of div 17 explants. The development of photoreceptor inner segments was normal; oil droplets were also noticed in the explants. Surprisingly, structures resembling photoreceptor outer segments were noticed in the explants even in the absence of pigment epithelium. Though the formation of outer segments was clearly indicated, they were spherically elaborated instead of forming lengthy structures. The membrane stacks were not organized but were diffusely arranged in comparison to their regular arrangements of stacks in vivo (see Fig. 8).

Discussion

We have highlighted a simple but most effective *in vitro* model system to culture embryonic chicken retina for extended periods. *In vitro*, retinal tissues could be cultured either as monolayers, dissociated cells, slices, reaggregates or explants. Monolayer retinal cultures are quite useful in studying the expression of proteins by individual cells which lack interactions with other cell types, e.g. the enzyme glutamine synthetase of Müller glia in chick retina could be precociously induced by corticosteroids in the absence of cell-cell interactions (Dutt and Reif-Lehrer, 1980). Also, dissociated cell cultures are useful in conducting cell proliferation or apoptotic assays (Gustmann and Duenker, 2010). Reaggregation cultures of embryonic chick retina are of great importance in 3D tissue reconstruction (Layer and Willbold, 1994) and tissue engineering (Layer et al., 2002; Rieke et al., 2008). Though the above-mentioned culture systems have their own significance and advantages, explants could be considered to mimic more of an *in vivo*-like environment under favourable conditions because of their tendency to preserve the retinal cytoarchitecture *in vitro*. The first main difference in our culture system compared with the already existing systems is the absence of any supporting materials or substrata. Though a number of substrata, like plasma or fibrin clots (Barr-Nea and Barishak, 1970; Hoff et al., 1999), millipore filters (Caffé et al., 1989), etc. were used as supporting materials for the explants and did not interfere with the growth or development of the latter, we present a more natural environment to the retinal tissues in our system. The second difference is that the explants undergo continuous orbital rotation at an optimal speed of 72 rpm in 35 mm culture dishes. Thereby, the explants were continuously exposed to the media due to their free floating status. The wide space available in the culture dish supported the growth of tissues in a 3D-like environment.

We used retinas from E6 chick embryos which are immature at this stage. At the beginning of the culture, the explants represent merely neuroblastic tissues without any cell layers being formed. After two days of culture, the explants started to develop and differentiate very much like that of *in vivo* retina. We used whole retinal tissue parts as explants instead of slices. Adding to the above mentioned advantages, such whole retinal parts as explants serve as excellent model systems in following the developmental and differentiation pattern of all retinal

layers. In particular, the formation of the inner plexiform layer, and differentiation of specific cell types, contributing to IPL formation, is a feature which might be difficult to follow in other culture systems. During the course of several culture days, the explants gradually reached a cytoarchitecture that is highly comparable to *in vivo* retina. Above all, the tissues maintained the retinal structure through 2-3 weeks without any considerable distortion. Not surprisingly, the retinal ganglion cells (RGCs) are lost even before 2 days of culture due to severing of the optic nerve that also resulted in thinning of the IPL. *In vivo*, a population of amacrine cells termed cholinergic type II amacrine cells or displaced amacrine cells (DACs), migrate towards the GCL forming the IPL. Interestingly, in explants the DACs migrated, forming an IPL even in the absence of the GCL.

We also used embryos from developmental stages at E10 and E15 to produce explants and to study the efficiency of these explants in forming higher order structures. Explants from E10 were able to produce organized structures that were comparable to explants from E6, but those from E15 were not able to survive in culture for more than a week (only statistical data). A simple explanation could be due to the large size of retinal tissue that had to grow in relatively smaller space.

Müller cell differentiation in explants is observable by 6 days *in vitro* (data not shown), the stage that is equivalent to E12 *in vivo*, as judged by immunostaining for glutamine synthetase, a marker for glial differentiation. *In vivo*, Müller cells mature only after the 15^{th} embryonic day. Therefore, the explants displayed an advanced differentiation pattern *in vitro*. The possible explanation could be that some available growth factors or proteins present in the serum might induce an early differentiation. In monolayer cultures and retinal explants, corticosteroids accelerate the differentiation of Müller cells by precociously triggering the enzyme glutamine synthetase (Dutt and Reif-Lehrer, 1980; Linser and Moscona, 1981). This type of early differentiation was also observed in retinal reaggregated spheres (own observation).

We found the expression of neurofilaments in explants to be surprising as they are specific intermediate filament proteins expressed only at the onset of ganglion cell differentiation (McCabe et al, 1999). However, recent studies have shown that type II cholinergic amacrine cells in the chick transiently express neu-

rofilaments (Stanke et al, 2008). Their transient expression appears to be prolonged in our explants cultures.

The most spectacular and innovative advantage of the explant system is the differentiation of photoreceptors, including that of structures resembling outer segments. Most of the earlier culture systems failed to support the development of photoreceptor outer segments (Araki et al., 1987). The presence of pigment epithelium was required along with the explants to support the formation of outer segments (Caffé et al., 1989). Our simple explant system supports the formation of photoreceptor outer segments and can maintain its laminar structures outstandingly well for 2-3 weeks even in the absence of pigment epithelium. Electron microscopic studies better resolved the onset of formation of outer segments in explants. Though outer segments were clearly formed, they had not achieved their typical long cylindrical morphology, but appeared elaborated. In addition, the inner membranes were not stacked in parallel, but rather were aligned in confluent long membrane sheets (see Fig. 8). Earlier studies from retinal rudiments of *Xenopus laevis* have shown similar elaborated outer segment structures in the absence of pigment epithelium (Steimke et al., 1994). Similar to the advanced differentiation of Müller cells, the photoreceptors also developed much earlier *in vitro* by div 4 onwards (data not shown). These observations could provide an important link of association between both cell types (Wang et al., 2005). In retinospheres, delaying of Müller cell differentiation by glial specific toxins delayed the differentiation of photoreceptors (own observations).

In conclusion, our simplified retinal explant culture system promises to provide a well organized experimental model that could be used to follow and/or manipulate the developmental processes over longer periods, especially the development of photoreceptors and their outer segments *in vitro* without any substrata. Also, our system could become highly useful for studies on the action of growth factors as well as the toxicological effects of foreign agents.

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Chapter IV

Manuscript in preparation

Both ganglion cells (GCs) and amacrine cells (ACs) play important roles in the development of the IPL in the retina. The migration of type II SACs towards the GCL might be environmentally influenced by the GCs. In this chapter, using the explants system and 3D retinospheroids, I show that the development of the IPL and its stratification occurs in the absence of GCs.

Development of a stratified inner plexiform layer (IPL) is independent of ganglion cells in chick retinal explants and spheres

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Key words: acetylcholinesterase (AChE); Brn3a for ganglion cells; chick retina; choline acetyltransferase (ChAT); inner plexiform layer (IPL); IPL subband; starburst amacrine cells (SACs); retinospheroids

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Abstract

The inner plexiform layer (IPL) of the retina is a highly organized zone which is filled with processes from different types of retinal neurons. In chick embryonic retina, the initiation of the IPL begins as early as E8/E9. Since the ganglion cells are the first to differentiate followed by amacrine cells, these cells might play a vital role in the formation of the IPL. Two important types of amacrine cells called type I and II which are the only cholinergic cells are the early ones to differentiate closely following the ganglion cells. As the ganglion cells migrate towards the GCL, the type II cholinergic amacrine cells also displace to the GCL. Since the IPL is formed between the type I and type II cholinergic cells, and the latter being displaced in the GCL, we examined if the ganglion cells had any roles in the development of the IPL at early stages. Two different in vitro model systems were used in the present study. E6 chick embryonic retinae were used for the production of retinal explants and 3-dimensional retinospheroids. The cultures were maintained for approximately two weeks. Immunostainings on fixed sections of explants and spheres with Brn3a, a specific ganglion cell marker revealed that in both the in vitro systems the ganglion cells did not survive for more than two days. Further stainings with the main cholinergic markers, ChAT and AChE in addition to other IPL markers such as calretinin, neurofilaments and CRABP revealed that the IPL was formed even in the absence of ganglion cells, and interestingly, the type II cholinergic cells migrated towards the GCL like area which did not contain any ganglion cells and the cholinergic stratification developed intact.

Introduction

Any information regarding the visual activity is transmitted to the brain by retinal ganglion cells (GCs). Subsequently, other retinal neurons like bipolar cells (BPs), horizontal cells (HCs) and amacrine cells (ACs) also play major roles in transducing the visual signals from the photoreceptors. Both the GCs and ACs spread their dendrites which stratify in the inner plexiform layer (IPL) to form either ON or OFF sublaminae that is important for retinal physiology. The IPL is a highly organized synaptic zone where all information from the bipolar and amacrine cells is transmitted to the GCs (Drenhaus et al., 2003). To speak about ON and OFF sublaminae in vertebrate retina, the GCs and ACs which respond to increments or increase of light form the ON channel, while the cells which respond to decrements or decrease of light form the OFF channels (Famiglietti and Kolb, 1975, 1976).

The cholinergic stratification in the chick embryonic retina has been vastly investigated (Layer et al., 1998; Reiss et al., 1996). In chick retina, the development of the IPL begins from E7 onwards, while the cholinergic stratification begins from E9 onwards when the two types (type I and II) starburst amacrine cells become arranged in a pair-wise manner and their dendrites meet in the IPL (Spira et al., 1987; Prada et al., 1999). Acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) are two important markers that were used to decipher the main ON and OFF subbands. The OFF subband is formed by type I cholinergic amacrine cells which are situated in the border of the inner nuclear layer (INL) and the IPL, while the ON subband is formed by the dendrites of type II cholinergic amacrine cells which are displaced to the ganglion cell layer (GCL) and are found mixed with the ganglion cells.

The GCs are the first to differentiate in vertebrate retina and their dendrites stratify the IPL in two levels (Kahn, 1974; Snow and Robson, 1994). As the type II cholinergic amacrine cells are displaced in the GCL and are found in close vicinity of the GCs, the latter might have important roles in the differentiation and migration of the type II cells as well as the cholinergic stratification in the IPL. Since much is known about the cholinergic stratification in chick retina which contains a larger IPL area compared to mammalian retina and is also easily accessible, we used novel chick retinal explants and 3-dimensional retinospheroids which lack the GCs due to optic nerve transection in order to study the pattern of IPL devel-

opment and cholinergic stratification in the absence of the GCs. Other amacrine cell and ganglion cell markers such as calretinin, cellular retinoic acid binding protein (CRABP) and neurofilaments which also localize the different sublaminae within the IPL have been extensively used in this study to characterize the IPL stratification in vitro.

Materials and Methods

In vivo

Fertilized White Leghorn chicken eggs were purchased from a local farm and incubated at 37°C in a humidified chamber under occasional rotation. The stages of development were determined according to Hamburger and Hamilton (1951) and expressed as embryonic days (E) of development. Adult chicken were purchased and sacrificed by cervical dislocation.

Preparation of retinal explants

Explants were prepared as previously reported (Thangaraj et al., 2011). In short, eggs were opened at embryonic day 6 (E6) and the eyes collected in calcium free Hank´s Basal Salt Solution (HBSS). The central retina which was associated with the pigment epithelium was removed carefully without any contamination of the latter. The whole retinal cup was flattened with the ganglion cell layer facing upwards without any damage and cut into two equal parts and were transferred to 35 mm culture dishes containing DMEM (Dulbecco's Modified Eagle's Media) with a supplement of 10% fetal calf serum, 2% chicken serum, 1% L-glutamine, and 0.15% penicillin/streptomycin (all from Gibco, Berlin, Germany). The explants were cultured for at least 10 days with a routine media change once in every two days.

Preparation of retinospheroids

The retinas of E6 chick embryos were collected in F12 medium on ice. For dissociation, the retinas were treated with 1mg/ml trypsin for 10 min at room temperature. The tissues were then rinsed in F12 medium and gently dissociated into single cells with a fire polished Pasteur pipette in the presence of 0.05 mg/ml DNase. After a threefold wash in F12 medium, the cells were resuspended in culture medium (10% fetal calf serum (FCS), 2% chicken serum, 1% L-glutamine, 0.1% penicillin/streptomycin, 0.02mg/ml gentamycin in DMEM medium). Cells were suspended in such a manner that each plastic dish of 3.5cm contained two eyes in 2ml of culture medium. The cells were reaggregated by rotation on a gyratory shaker (72 rpm, 37° C, 95% air/ 5% CO_2). The medium was changed once in two days.

Fixation and cryosectioning

Whole eyes from different embryonic stages and adult chick, explants and retinospheres from different culture days were all fixed in 4% paraformaldehyde for 1 hr at room temperature or 24 hr at 4°C overnight. Fixed samples were washed in PBS and immersed in 25% sucrose and sectioned in a cryostat (Microm, Heidelberg, Germany) at 10-12 µm and mounted on frost-free or gelatine-coated glass slides. The sections were stored at -20°C until further use.

Immunohistochemistry

Sections of in vivo retina, explants and spheres were subjected to immunostaining. Prior to staining procedures, the frozen sections were dried on a heating plate at 37°C. The area around the sections was marked with a greasy liquid blocker (Roth, Karlsruhe, Germany) and the slides were pre-incubated in blocking solution which contains PBS, 3% BSA (Bovine Serum Albumin) and 0.1% Triton-X-100 for at least 30 min. 100 µl of primary antibodies which were diluted in the blocking solution were applied on the sections inside the area which was surrounded by the liquid blocker and then incubated for 90 min at room temperature or overnight at 4°C. In case of double staining, one polyclonal and one monoclonal antibody was mixed and applied together. The slides were washed thrice with PBS and then subjected to 100 µl of fluorescent conjugated secondary antibodies for 70 min at room temperature. After three PBS washes, the sections were treated with DAPI (Merck, Darmstadt, Germany) for 3 min in order to stain cell nuclei. The slides were rinsed in distilled water after a final wash in PBS and then dried on the heating plate and embedded in Kaiser's glycerol gelatine (Merck, Darmstadt, Germany). The details of antibodies used are as follows: Brn3a (1:1000), ChAT (1:1000), Calretinin (1:1000), CRABP (1:500), AChE (1:200), Neurofilaments (1:500), Cy3 (1:200) and Cy2 (1:200).

Cholinesterase staining

Acetylcholinesterase staining was performed by the Karnovsky and Roots technique (Karnovsky and Roots., 1964). In brief, the frozen sections were dried on a heating plate, incubated twice for 15 min in Tris-maleate buffer and treated with the reaction buffer (0.1M tris-maleate buffer, 0.1M sodium citrate, 30mM copper sulphate and 5mM potassium hexacyanoferrate).The reaction was per-

formed for 1 hr at 37°C with 6 mM acetylthiocholine iodide as substrate and 0.1 mM iso-OMPA to inhibit BChE. The reaction produced a brown insoluble precipitate (Hatchett´s brown) at the site of enzymatic activity which was visible under the light microscope.

Microscopy and Imaging

Immunostained *in vivo*, explant and spheroid sections were subjected to fluorescent microscopy (Axiophot, Carl Zeiss, Germany) which was connected to an automated digital camera (Axiocam). Imaging was done for each fluorescent channel separately and processed through extended focus imager, and all the pictures from different channels were combined as overlays and stored in zvi image format by Axiovision Basis software programme.

Results

Stratification of the inner plexiform layer in vivo

To demonstrate IPL stratification, three developmental stages that include an early E9, mid E13 and a matured adult chick retina had been immunostained for different markers. The ganglion cells (GCs) are the first to differentiate in all vertebrate retinas. Brn3a, a transcription factor which is specifically expressed in GCs was used as a marker for differentiating GCs. Though the protein is not used to localize cell processes, it was used to observe the distribution of the ganglion cells. By E9, almost all the GCs had migrated to the GCL (Fig. 1a). By E13, there was no much difference in the number of GCs (Fig. 1b), which had considerably reduced in the adult retina (Fig. 1c). Since the GCs migrate towards the GCL, they might have important roles in the development of the IPL and its stratification.

Cholinergic amacrine cells which are otherwise stated as starburst amacrine cells (SACs) are of two types. Type I SACs are found in the INL/IPL border while the type II SACs are displaced to the GCL and found in close vicinity of the GCs. At E9, two ChAT+ bands were already observed in the IPL (Fig. 1d). By E13, the IPL had become wider, accompanied by further migration of the type II SACs. ChAT expression was weaker in the processes and the subband d formed by the former (Fig. 1e). In an adult retina, expression of ChAT again raised in type II cells and the two prominent cholinergic bands were well established (Fig. 1f).

AChE was also used as a reliable marker for stratification of the IPL. At E9, in spite of the two ChAT subbands formed, AChE was found to be expressed as a broad band, and had not yet stratified (Fig. 1g). Also more cells in the INL and GCL were AChE+. By E13, the broad band had resolved to two mild bands that would co-localize with the ChAT subbands (Fig. 1h). The number of AChE+ cells was reduced. In an adult retina, in addition to the main subbands, two milder AChE bands were observed in between (Fig. 1i). A similar pattern was observed in AChE activity by Karnovsky and Roots staining (Fig. 1j-l).

Further, IPL stratification was demonstrated by using calretinin, a calcium binding protein expressed by GCs and non-cholinergic amacrine cell subtypes. At E9, two mildly stained bands were observed in the IPL.

The GCs strongly expressed the protein (Fig. 1m). By E13, GCs had less calretinin, but more INL cells expressed the protein.

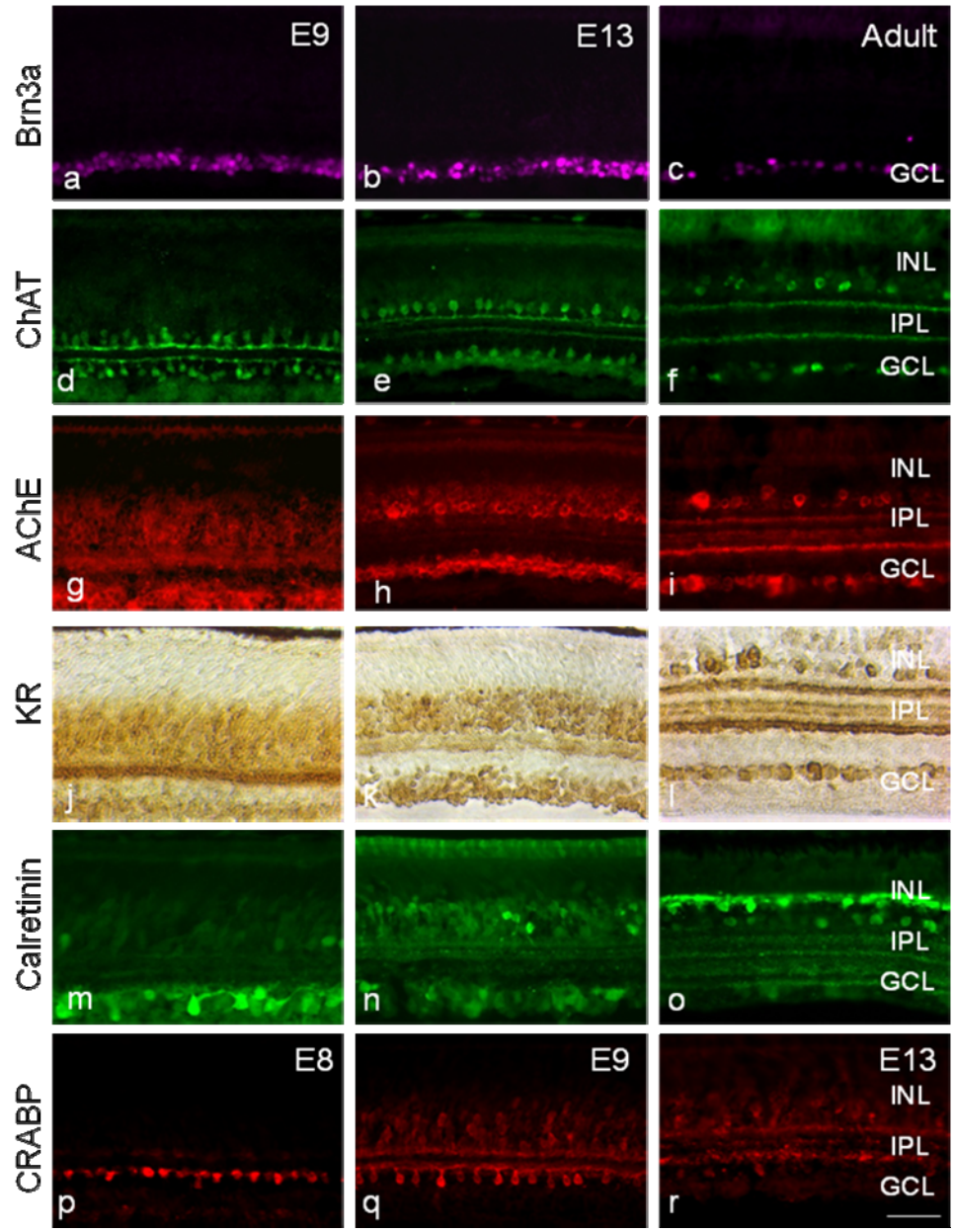

Fig.1: Immunodetection of IPL stratification in chick retina: (a-c) shows the ganglion cells immunostained by Brn3a (magenta) in E9, E13 and an adult retina, respectively. Similar developmental stages stained for ChAT (green) is shown in (d-f). IPL stratification begins at E9. At E13 (e), the subbands are prominent but the subband d had less ChAT accompanied by migration of type II SACs towards the GCL. In an adult retina (f), ChAT is again strong in both the subbands. The same sections stained for AChE (red) are shown in (g-i). Karnovsky and Roots staining for AChE enzyme activity also revealed a similar stratification pattern observed by AChE immunostaining (j-l). Further, stratification is shown by calretinin (green in a-o) and CRABP (red) in panels (p-r) immunostaining

Abbrev: AChE - acetylcholinesterase; ChAT – choline acetyltransferase; GCs – ganglion cells; GCL – ganglion cell layer; INL – inner nuclear layer; IPL – inner plexiform layer; SACs – starburst amacrine cells. Scale Bar = 50 µm.

Three bands were visible at this stage (Fig. 1n). In an adult retina, a very few INL cells expressed calretinin, but the protein was distributed in five subbands in the IPL (Fig. 1o). Cellular retinoic acid binding protein (CRABP) is strongly expressed in type II SACs at E8 (Fig. 1p). At E9, two subbands were observed in the IPL that would localize with the ChAT subbands (Fig. 1q). At E13, the type II SACs had already down regulated the expression of CRABP, consistent with earlier reports (Stanke et al., 2008). The protein was still distributed in three layers in the IPL (Fig. 1r).

Development and stratification of the IPL in retinal explants

Retinal explants prepared from E6 chick embryos provided a near in vivo like tissue model in studying the development and stratification of the IPL. As mentioned above, the GCs are the first to differentiate in the retina.

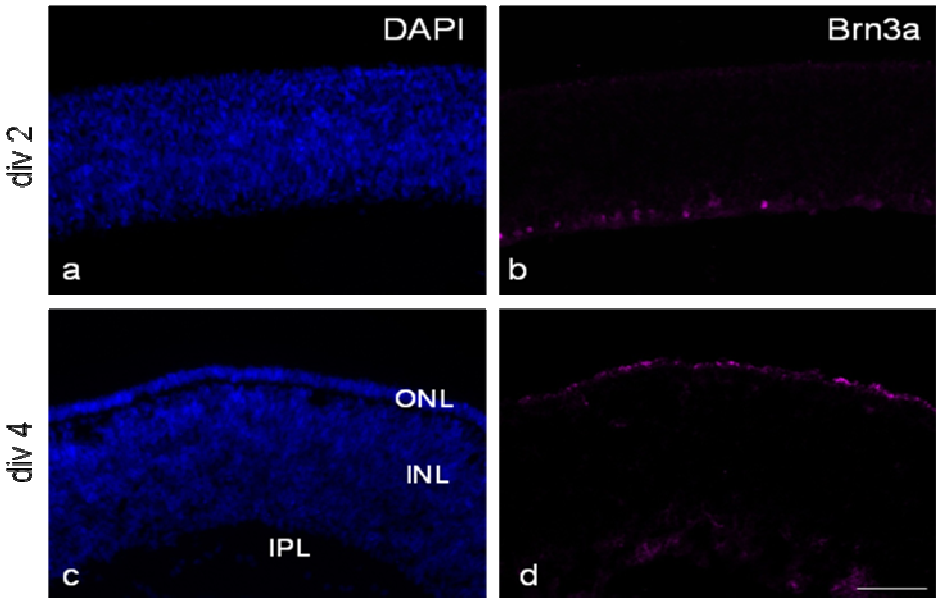

Fig.2: Ganglion cells do not survive in retinal explants: E6 chick embryonic retinae were used to produce explants in culture. Sections of explants cultured for 2 and 4 div were stained for DAPI to observe cell nuclei. At div 2 (a), the explants were almost neuroblastic and after 2 days, different cell layers were clearly visible (c). (b and d) are the same sections stained for ganglion cell specific marker Brn3a (magenta). At 2 div (b), a very few ganglion cells could be seen at the basal surface of the explants. At div 4, not even a single ganglion cell could be observed, owing to optic nerve transcetion. **Abbrev:** INL – inner nuclear layer; IPL – inner plexiform layer; ONL – outer nuclear layer. Scale bar = 50 µm.

Immunostaining for Brn3a showed that the GCs did not survive for more than two days in vitro. By two days in culture, the explants were mere neuroblastic without

any cell layers (Fig. 2a) and a very few ganglion cells were observed in the basal portion of the explants (Fig. 2b). After 4 div, all the retinal layers were clearly formed but no GCs were found owing to optic nerve transection (Fig. 2c, d).

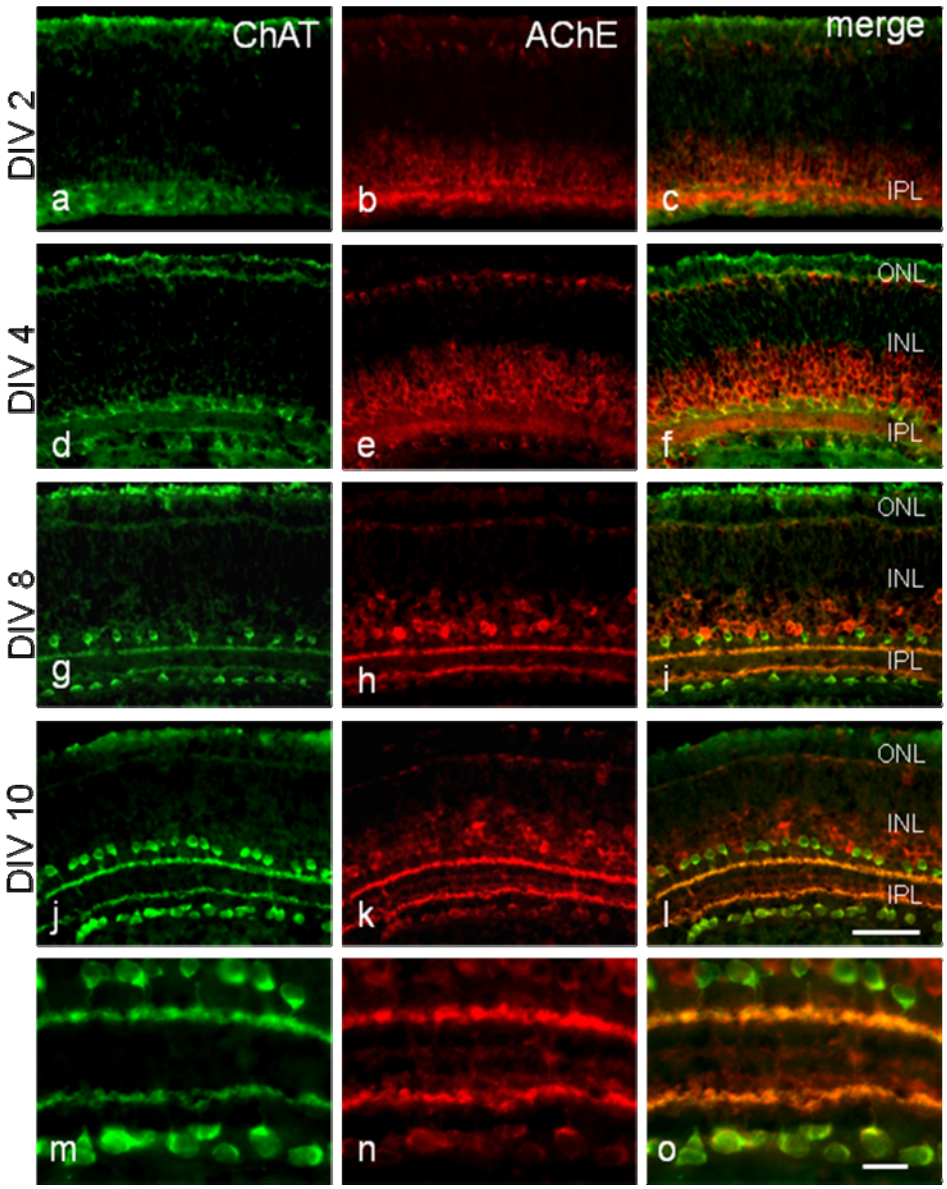

Fig. 3: Cholinergic stratification in explants: Retinal explants of div 2-10 stained for ChAT and AChE to follow IPL stratification is shown. (a-c) At 2 div, few ChAT$^+$ cells are found in the presumptive IPL (a), while AChE is already observed as a continuous band (b). The merge of two channels is shown in (c). By div4, two types of SACs are formed as observed by ChAT staining (d). Two ChAT subbands are observed. AChE at this stage is still a wide band (e and f). Note a large number of AChE+ cells in the INL as well as a single layer of cells in the border of the ONL. After 8 div, two prominent cholinergic subbands which co-localized both ChAT and AChE are observed (g-i). At 10 div, in addition to the two main subbands, two weaker AChE bands could be observed in between (j-i). The AChE+ cells in the INL have reduced in their numbers. (m-o) are higher magnification of (j-l) showing the IPL and the subbands.
Abbrev: INL – inner nuclear layer; IPL – inner plexiform layer; ONL – outer nuclear layer. Scale bar = 50 µm (a-i) and 20 µm (m-o).

Cholinergic stratification occurs in the absence of GCs in explants

The starburst amacrine cells (SACs) are found in close vicinity to the ganglion cells during early development of the retina. In vivo, type II cholinergic amacrine cells migrate in the IPL towards the GCL. As the ganglion cells do not survive in the explants even till the IPL is initiated, these explants proved to be good in vitro model system for the development of IPL and cholinergic stratification. ChAT and AChE immunostaining was performed on explants sections from div 2 to 10 in order to follow the development of the cholinergic stratification. At 2 div, IPL was not yet formed clearly and the two types of SACs could not be differentiated atleast by ChAT staining. But AChE was already observed as a band in the presumptive IPL (Fig. 3a-c). After 4 div, the type II SACs still migrated even in the absence of a true GCL and two prominent ChAT subbands which were separated by a wide band of AChE were observed in the IPL (Fig. 3d-e).

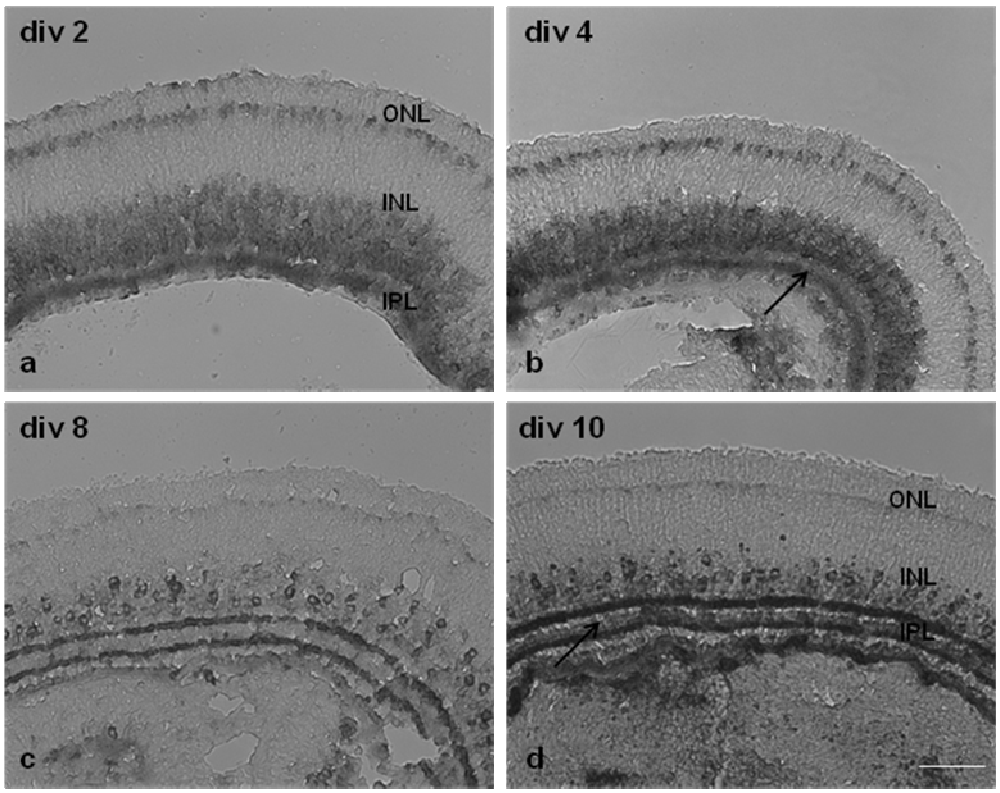

Fig. 4: Retinal explants stained for AChE activity by Karnovsky and Roots technique. At 2 div, AChE is found distributed in the cells of the INL and as a continuous band in the presumptive IPL (a). After 4 div, a wide band is observed in the IPL (arrow in b). Note two resolved bands in the IPL by div 8, accompanied by a reduction in the $AChE^{+}$ cells in the INL (c). At 10 div, the two subbands are strongly stained, and in addition two weak bands are observed in the IPL (arrow in d). Scale bar = 50 µm.

Like in vivo, increased numbers of AChE+ cells were observed in the IPL which almost gave a wire fall like appearance. By 8 div, the wide AChE band had resolved to two bands co-localizing with the ChAT subbands (Fig. 3f-g). After 10 div, in addition to the two prominent cholinergic subbands, two weak AChE bands were observed in between the subbands in the IPL (Fig. 3h-i). Imitating in vivo, the numbers of AChE+ cells in the INL also had considerably reduced in the explants (Fig. 3j-o). Karnovsky and Roots staining for AChE activity in explants also showed a similar pattern of distribution of amacrine cells in the INL and IPL as well as in the IPL subbands (Fig. 4).

In addition to cholinergic stratification, other marker proteins such as calretinin, CRABP and neurofilaments which are also observed in different substrata in the IPL were used to characterize the IPL stratification in retinal explants following the elimination of the GCs. Two stages of in vitro explants were used. At 4 div, two strong bands of CRABP were observed in the IPL (Fig. 5a).

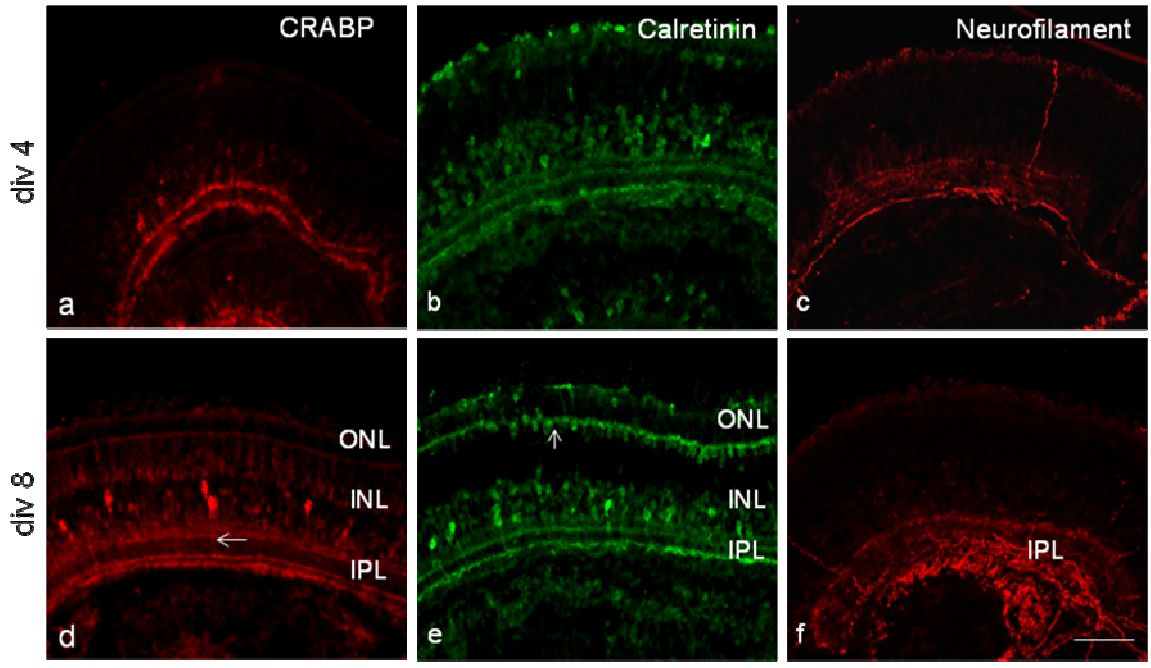

Fig. 5: Characterization of IPL stratification in explants with different markers: Div 4 and 8 explants stained for CRABP, calretinin and neurofilaments (a-e). Note that the CRABP staining in div 4 explants (a) is well organized by 8 div (d). Long processes from an INL cell passes through the IPL (arrow in b). More cells in the outer part of the INL are mildly stained. Calretinin subbands are similar at both stages (b and e). A row of cells is observed at the ONL border (arrow in e). Neurofilament staining was weak in the IPL of div 4 explants (c), but was still found in div 8 explants even in the absence of ganglion cells (f). Scale bar = 50 µm.

Also two well established calretinin bands were formed even in the absence of the ganglion cells (Fig. 5b). Interestingly, neurofilament staining was also observed in the IPL (Fig. 5c). As the neurofilaments are specific proteins of the GCs, their presence in the IPL of retinal explants was a surprise. After 8 div, more cells became mildly stained for CRABP in the outer half of the INL (Fig. 5d). A few strongly stained cells were observed in the lower half of the INL. Long processes could be noticed that passed through the entire length of the IPL (arrow in Fig. 5d). Similarly, calretinin also was observed in two bands in the IPL even after 8 div (Fig. 5e). A separate layer of cells at the border of the ONL were also strongly calretinin+ and by at least the position of these cells, they could be recognized as horizontal cells (arrow in Fig. 5e). Even after 8 days of culture, neurofilament immunoreactivity was observed in the IPL (Fig. 5f). Though the staining was present, the substrata were not clearly formed in the IPL in explants when compared to that of in vivo (see Fig. 6a, d in chapter III).

Development of IPL and stratification in 3D chick retinospheroids

Retinae from E6 chick embryos were enzymatically dissociated and cultured under constant rotation to form histotypical retinospheroids. Under culture conditions, the single cells reaggregated to form clusters. As the development proceeded, spheres showed a remarkable change in their morphology (Fig. 6a-d). The most interesting and specialized characteristics of these reaggregated spheres is sorting out of “like cells”. The inner retinal cells sorted out to form IPL-like areas, while the photoreceptors sorted out to form rosettes (see Fig. 6, triangle and arrow in 6f-h, respectively). Similar to retinal explants, the GCs were fully absent after two days in culture. Brn3a-staining detected only very few cells at 2 days of culture. These cells appeared to be randomly distributed, as the spheres were not fully organized (Fig. 7).

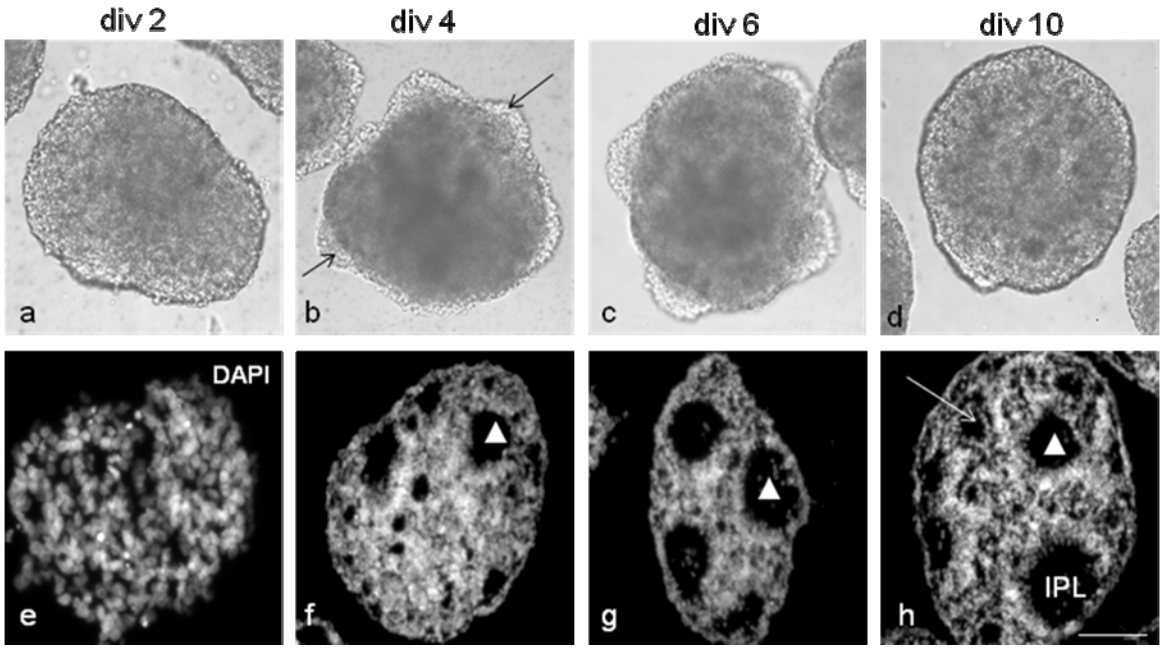

Fig. 6: Development of retinospheroids: Light microscope pictures of whole-mount spheres in culture (Fig. 6a – d). Initially, the spheres were nearly circular in shape at around 2 div (a). As the cells began to sort out, bulges at certain areas appeared at around 4 div and stayed up to 6 div (arrows in Fig. 6b, c). By 10 div, the spheres again reached a near-circular morphology (Fig. 6d). DAPI staining of cryosectioned spheres are shown in (e-h). Initially, the cells reaggregated to form a spherical structure (e). Around 4 div, the cells are tightly packed all over the spheres. Some cell-free spaces are formed which corresponds to an IPL-like area (triangle in f, g and h). At 6 div, the IPL-like areas are well developed, at least by surface area. A few cells are observed in the IPL. By 10 div, the spheres reached near-circular morphology. A rosette is observed which is devoid of any cells in its centre (arrow in h).
Abbrev: div – days in vitro, IPL – inner plexiform layer. Scale bar = 50 µm.

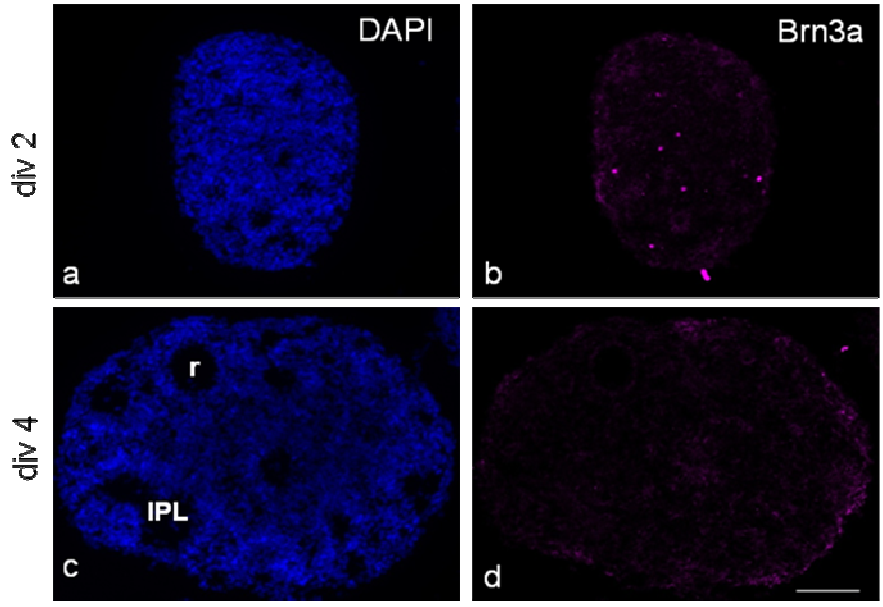

Fig. 7: Ganglion cells do not survive in retinospheroids: Sections of spheres stained for Brn3a (magenta). At 2 div, the spheres are not yet organized as revealed by DAPI staining (a). A very few Brn3a+cells are found scattered in the sphere (b). At 4 div, the spheres began to organize to form histotypical structures. Note a rosette (denoted by r) and an IPL like area being formed (c). At this stage, not even a single Brn3a+ cell could be found (d). Scale bar = 50 µm.

Stratification of IPL in retinal spheroids

As far as the cholinergic stratification is concerned, the two main cholinergic subbands were still formed in the spheres even in the absence of ganglion cells, as revealed by ChAT and AChE immunoreactivity. Interestingly, the type II SACs migrated into the IPL-like areas in the absence of any environmental cues from ganglion cells (Fig. 8).

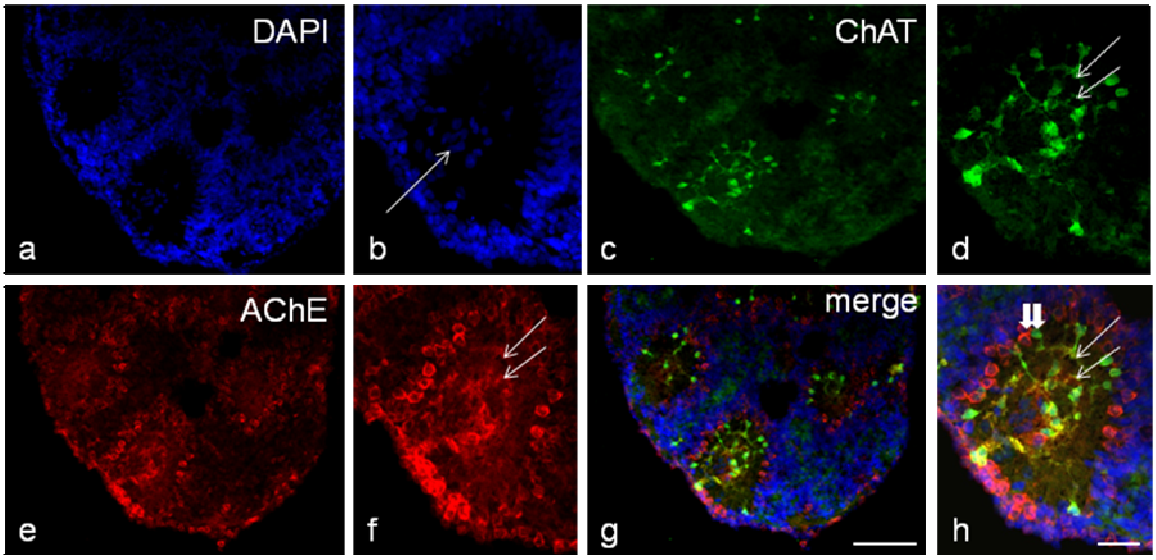

Fig. 8: Cholinergic stratification in retinospheres: 8 div retinosphere showing the IPL and cholinergic stratification. The cell nuclei are stained with DAPI (blue). Note an IPL with a few type II SACs in the middle (a). A higher magnification of the type II cells (arrow in b) and the IPL like area is shown (b). The IPL subbands are detectable as revealed by ChAT (green) and AChE (red) immunostaining (c-h). The subband a is clearly formed but the subband d is formed around the type II SACs (arrows in d, f and h). AChE staining is shown in a separate channel in e; f is a higher magnification of e. A merge of all channels is shown in g and h. Type I SAC in the border of the IPL is neighbored by intensely stained non-cholinergic AChE+ cell (block arrows in h, a much higher version of the picture is shown just below). Scale bar = 50 µm (a, c, e and g); 20 µm (b, d, f and h).

The type I SACs were found arranged in the border of the IPL-like areas and the unorganized cellular areas. Like in vivo, each type I SAC was neighbored by non-cholinergic, strongly AChE stained cell. Type II SACs were found in the middle of the IPL-like areas. Karnovsky and Roots staining also revealed the two cholinergic subbands in the IPL. At 2 div, AChE activity was minimal in the whole sphere. After 4 days of culture, AChE activity was found in INL cells surrounding the IPL-like areas and also in the type II cells in the IPL (Fig. 9). The main cholinergic subbands were detectable by AChE reactivity.

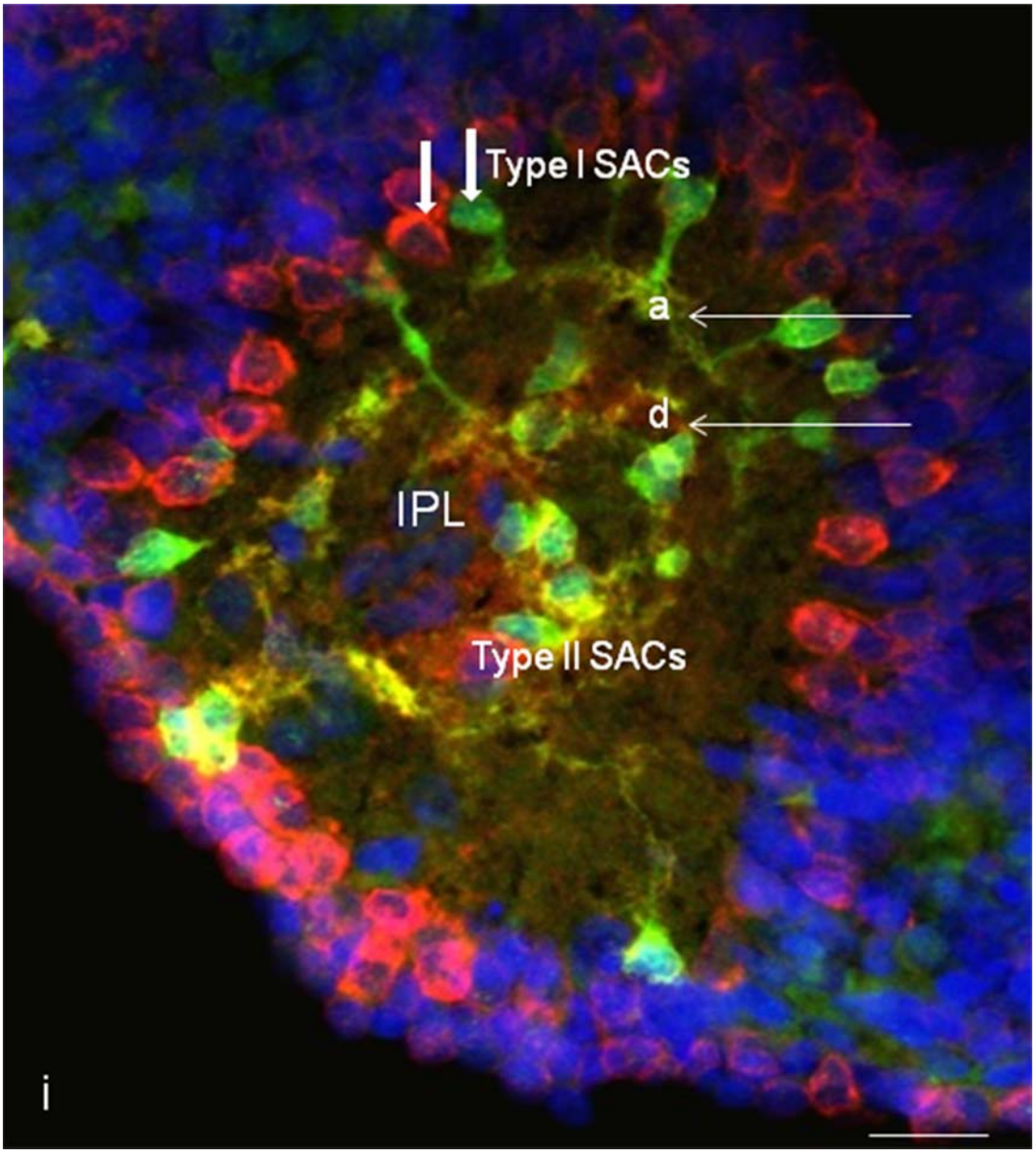

Fig. 8i: A larger version of figure 8h. The type I SACs are found in the border of the IPL, while type II SACs is found in the IPL. The processes of type I SACs form the subband ‘a’ and the processes of type II SACs form the subband ‘d’ (arrows). Note the type I SACs in the border of the IPL is neighbored by cells intensely stained by AChE which do not contain ChAT (block arrows).

Stratification of the IPL was further characterized by other markers such as calretinin and neurofilaments (Fig. 10). During the early culture periods, both the proteins were expressed in cells that were randomly arranged

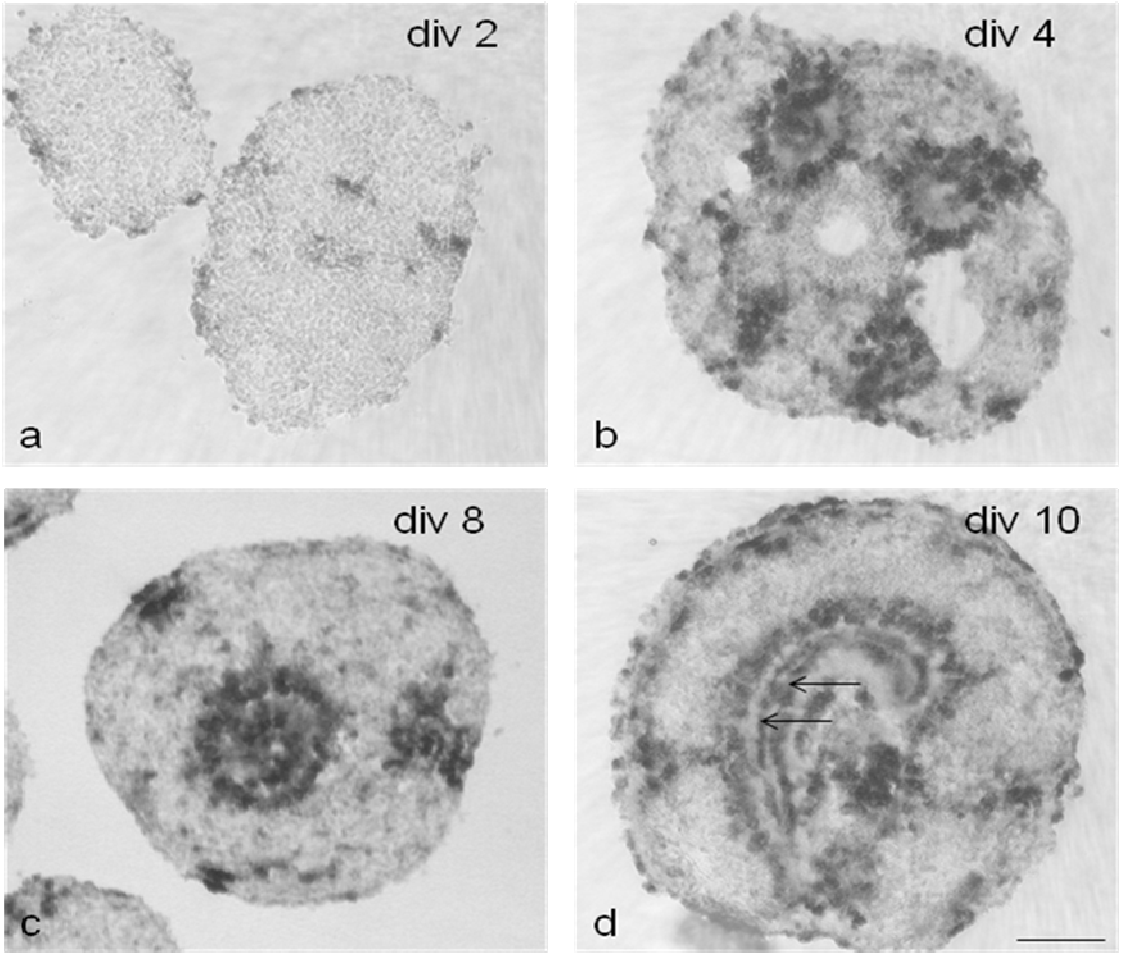

Fig. 9: AChE activity (K&R) staining of retinospheres: At 2 div, AChE activity is very low (a). At 4 and 8 div, the cells surrounding the IPL-like areas and the type II SACs in the IPL are all AChE+ (b and c). Note the two cholinergic subbands at 10 div (arrows in d) that are revealed by AChE acivity. A few more AChE+ cells are also observed in non-organized areas. Scale bar – 50 µm.

By 4 div, IPL like areas were formed but unlike in vivo and explants, continuous bands were not observed in the retinospheres. Again in the absence of ganglion cells, the GC specific protein neurofilament was still expressed in the IPL but the protein was not localized to any cells. Moreover the expression was reduced by 4 days. By 6 div, calretinin was observed in numerous cells in the spheres which might be different population of amacrine cells and horizontal cells. Processes from some of the cells situated around the IPL like areas reached the IPL where they formed one or two mild bands (arrow in Fig. 10h).

Interestingly, the expression of neurofilaments again increased and was observed as continuous bands in the IPL. However, the expression was not observed in any cell bodies (arrow in Fig. 10i).

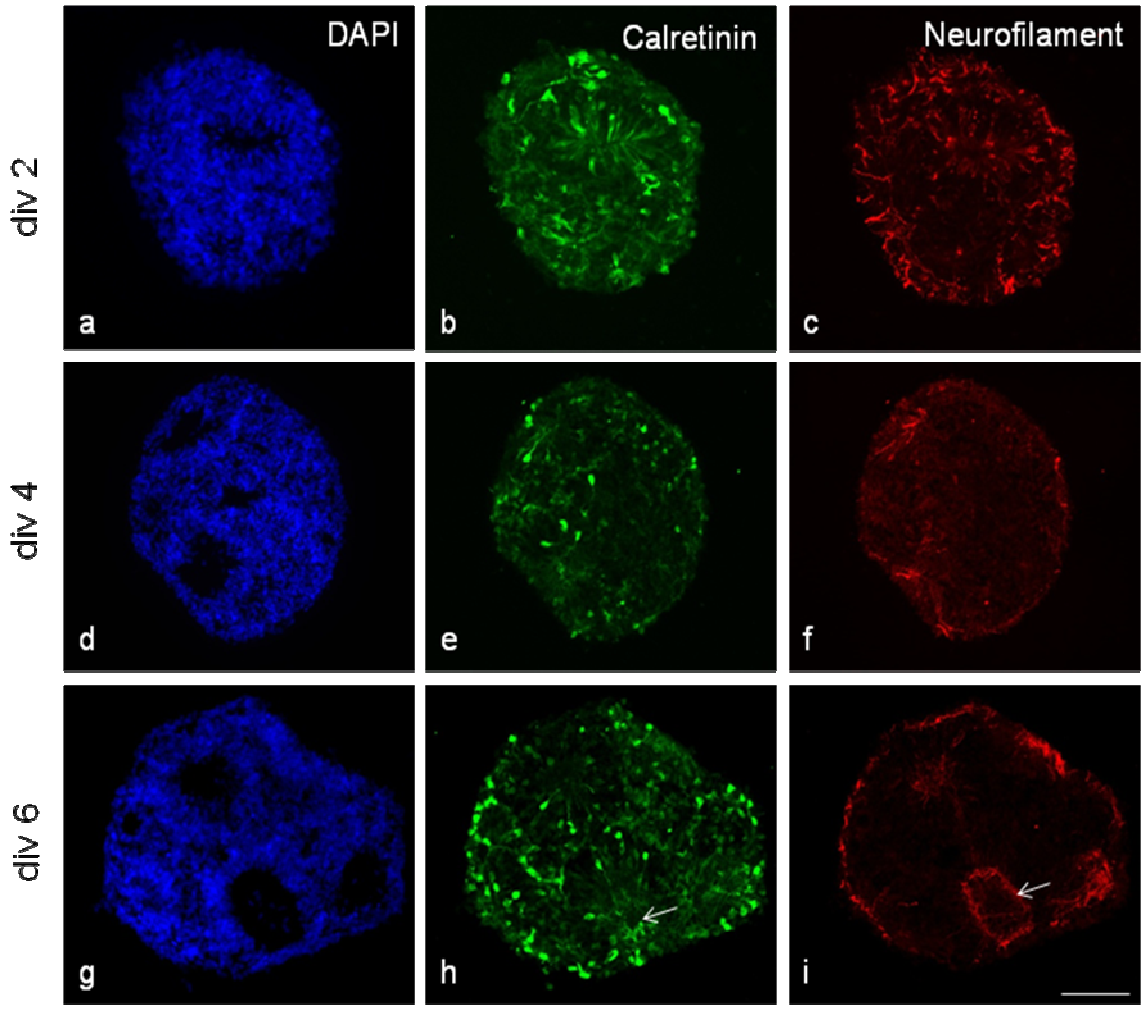

Fig. 10: Expression of calretinin and neurofilament in the IPL: In order to follow stratification of the IPL in spheres, sections immunostained for calretinin and neurofilaments are shown. At 2 div, the IPL is not yet completely formed (a) as shown by DAPI staining (blue). Cells around the forming IPL strongly expressed calretinin whose processes are directed towards the IPL (arrow in b). Neurofilament immunoreactivity is observed as short processes over the spheres (c). At 4 div, IPL-like areas are larger when compared to 2 div (d). The number of cells expressing calretinin has decreased (e). Expression of neurofilaments has also decreased in the spheres (f). By 6 div, IPL-like areas are formed even better (g). Both calretinin and neurofilaments expression has again increased (h and i). Long processes from cells surrounding the IPL-like areas projected into the IPL, forming mild bands (arrow in h). Neurofilament staining was stronger in the IPL-like areas (arrow in i) but was not observed in any cell bodies. Scale bar = 50 µm.

Discussion

The inner plexiform layer of the vertebrate retina is a highly organized synaptic layer where the processes of various cell types of the INL are interconnected with the processes of ganglion cells (Reiss et al., 1996). In chick retina, the IPL development begins shortly after the appearance of the two types of SACs adjacent to the vitreal surface at E6 (Millar et al., 1985; Millar and Chubb, 1987; Spira et al., 1987; own observation; see chapter I). The beginning of the development of the IPL is accompanied by the migration of the type II SACs as early as E8 while its initiation might be triggered by a narrow band of AChE at E7 that separates the two types of SACs (discussed in chapter I). The different amacrine and ganglion cell types stratify the IPL at different layers forming the sublayers (Drenhaus et al., 2003). The cholinergic subbands “a” and “d” along with the other sublayers form the main basis of OFF and ON retinal physiology (Layer et al., 1997).

Based on our in vivo data, we report that the cholinergic stratification occurred at E9 as could be observed by the appearance of the two ChAT bands in the IPL followed by AChE bands that co-localized with the former by E11-13. Two more AChE bands were noticed in between the main subbands, consistent with earlier reports (Reiss et al., 1996). Calretinin, a calcium binding protein which is expressed in different types of amacrine and ganglion cells (Ellis et al., 1991) and cellular retinoic binding protein (CRABP) were used as markers to characterize the IPL stratification and to compare it in vitro. Though much is known about the IPL development, the relationship between the IPL development and ganglion cells is still uncertain. Since the GCs are in close vicinity of the migrating SACs, their involvement in the development of the IPL and its stratification was studied by employing novel explants and 3-dimensional culture systems that lack the GCs due to optic nerve transection.

Development of the IPL and its stratification occurs in the absence of GCs in retinal explants

Retinal explants from E6 chick embryos proved to be a suitable in vivo like model system to follow IPL development. We used E6 retina, as the development of the tissue is only at its initial stage. Immunostaining with GC specific protein Brn3a showed that the GCs never survived for more than two days in vitro. The

IPL was not developed in 2 div explants but after 4 days, IPL was well formed along with the two ChAT bands even in the absence of GCs. Interestingly, the type II SACs even migrated to a certain distance, thereby increasing the width of the IPL. The guiding signals for the migration of these cells remain unknown. Other possibilities might be that the signals had been already passed on by the GCs to the amacrine cells very early before their extinction. In rats and ferrets it was shown that ACs develop and maintain ON and OFF stratification patterns after GCs had been experimentally degenerated (Günhan-Agar et al., 2000, Williams et al., 2001). But the possibilities of stratification were much higher in these experiments and the role of GCs could not be emphasized as the cells were targeted much later after the formation of the IPL. In *lak* (gene encoding *Atonal* homologue 5 (Ath5), a basic-helix-loop-helix-transcription factor required specifically for the genesis of GCs) mutant zebrafish, which do not possess any GCs, the development of the IPL was delayed. However, IPL sublamination still occurred with local disruptions (Kay et al., 2004). Though the staining of calretinin was not localized as five sublayers like that of in vivo (Fig. 1o), the explants showed at least two bands with CRABP and calretinin. Surprisingly, the IPL in explants stained for neurofilaments as well which is a GC specific protein (discussed in chapter III) in the absence of ganglion cells.

Are the type II SACs programmed to displace into the IPL?

The question that remains is which signalling cues drive the orientation of the type II SACs towards the GCL. Though in retinal explants, the type II SACs displaced themselves even in the absence of GCs at div 4, the possibility of early signalling by the GCs cannot be entirely ruled out, as discussed above. In order to answer this question, we used 3-dimensional retinospheroids where the retinal tissues from E6 chick embryos are dissociated into single cells and allowed to reaggregate under constant rotation (Layer and Willbold, 1994). Thereby, the cell-cell contacts between amacrine and ganglion cells were lost during dissociation. Moreover, a very few GCs were observed at early div 2 when the spheres were not even organized into histotypical structures.

Interestingly, after 2 days of culture cells began to sort out in the spheres, resulting in histotypical structures that presented photoreceptor rosettes and IPL-like areas. The type II SACs still migrated into the IPL-like areas and the cho-

linergic subbands were formed even in the absence of GCs and also after dissociation of the tissue. This kind of organization prompted us to think whether the type II SACs is programmed since their birth to displace or migrate into the IPL even in the absence of GC signalling. Like retinal explants, the retinospheroids also expressed neurofilaments which is a GC specific protein. Though differentiating type II SACs transiently express neurofilaments (Stanke et al., 2008), we did not expect any staining at later stages of culturing.

Based on the observations, it could be interpreted that amacrine cells seemed to be more dispensable for the stratification of the IPL than the ganglion cells. However, some of the targeting sublayers for the bipolar cells which stratify during later developmental stages might have been lost due to the absence of the guiding GCs. A mouse model which lacks bipolar cells also showed normal amacrine cell sublamination (Green et al., 2003).

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Chapter V

Manuscript in preparation

In addition to SACs, early Müller glial cells (MCs) might provide important guiding factors during development and stratification of the IPL. To test this hypothesis, the Müller glial cells were targeted in 3D retinospheroids by the specific gliotoxin DL-alpha aminoadipate. In this chapter, I discuss the importance of Müller glial cells in IPL as well as other developmental roles on retinal neurons.

Analysis of IPL formation in retinal spheroids: the gliotoxin DL-alpha-aminoadipate (AAA) induces a cell type shift towards inner retinal cells, while decreasing photoreceptor differentiation

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Key words: alpha aminoadipate; chick retina; inner plexiform layer (IPL); IPL sub-band; Müller glial cells; starburst amacrine cells (SACs); retinospheroids; photoreceptors

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Abstract

The possible role of a sub-toxic effect of DL-alpha aminoadipate, a specific Müller glia toxin during early development of the retinal neurons was investigated in 3D-reaggregated histotypic spheres, derived from dispersed cells of the E6 chicken embryo retina. Supplementation with a final concentration of 0.4 mM DL-alpha-aminoadipate reduced the expression of Müller glia-specific enzyme glutamine synthetase in the IPL-like areas, suggesting a delay or disturbance in the complete differentiation of the glial cells. TUNEL histochemistry showed no difference between control spheres and spheres treated with toxin from the beginning of culture, indicating that the toxin is not fatal to the Müller cells during early development. Immunochemical studies showed that DL-alpha aminoadipate increased the number of inner retinal cells, especially amacrine cells which was confirmed by different amacrine cell markers. The treatment increased the expression of acetylcholinesterase which is mostly restricted to different types of amacrine cells. Furthermore, an increase in the amacrine cell-specific proteins axonin1 and Pax6 also confirmed the increase in the cell type. In contrast, differentiation of both r/g cones and then rods, as detected by CERN-906 and CERN-901 antibody binding, respectively, was found drastically reduced, while the photoreceptor precursors, as detected by the expression of visinin, remained unaffected by the treatment, complying with other earlier reports suggesting the importance of Müller glial cells in photoreceptor differentiation.

Introduction

The embryonic development and the organization of the CNS, especially the brain parts arise from complex mechanisms that include proliferation and growth of neuroepithelial tissue, migration, dispersion, final destination of neurons, formation of synapses etc. The neural retina is one of the most suitable and easily accessible parts of the brain that is being used in order to study these complex mechanisms (Dowling, 1987). Not only because of its easy accessibility, but also due to its relatively simple organization, when compared to other brain parts, has made the retina a powerful model system to study neural development (Willbold and Layer, 1998).

The adult retina is composed of three nuclear layers containing a limited number of neuronal cell types: photoreceptors in the outer nuclear layer (ONL); horizontal, bipolar and amacrine cells in the inner nuclear layer (INL) and ganglion cells along with displaced amacrine cells in the ganglion cell layer (GCL) and two plexiform layers: outer plexiform layer (OPL) and inner plexiform layer (IPL). In addition to the neurons, the only glial cells available in the bird´s retina are the Müller glial cells which play very important roles in neuronal migration, neurite outgrowth and cellular differentiation (Willbold et al., 1997). It is widely accepted that both the neurons and Müller glial cells share a common progenitor. The question of how each individual cell is destined to its final fate and location to fit into the retinal architecture and circuitry is an interesting area of research. Though genetic and cell-intrinsic factors are believed to be important in cell fate determination (Watanabe and Raff, 1990), other micro-environmental cues could also have major impacts at least by cell-cell signaling (Turner and Cepko, 1987).

Of the many interesting characteristics of Müller glial cells, the most remarkable feature is their plasticity. Due to this remarkable ability, Müller cells can re-enter the proliferation cycle and develop into neurons after a retinal injury (Burke and Smith, 1981). As Müller cells are known to possess numerous receptors including epidermal growth factor (EGF) receptors (Gospodarowicz et al., 1986), fibroblast growth factor (FGF) receptors (Kinkl et al., 2001), nerve growth factor (NGF) receptors (Chakrabarti et al., 1990) etc., they also react to these growth factors when supplied in vitro. Recent studies have shown that in the presence of bFGF alone or in combination with insulin, Müller glial cells re-entered the proliferative phase and developed into neurons (Fischer et al., 2002).

In vitro, bFGF induced Müller cell proliferation but delayed photoreceptor differentiation and reduced cellular apoptosis (Frohns et al., 2009).

Though Müller glial cells are believed to be the last born cells during the course of retinal development, there are increasing evidences that Müller cells are present from the very early beginning of retinogenesis (Willbold and Layer, 1998). The early Müller cells or immature precursors might provide important environmental cues to the developing neurons by providing guidelines to cells to migrate, extend neurites or find the final destination of the cells. It has been already shown that early disturbances in Müller cells results in disturbed migration pattern of displaced amacrine cells (Prada et al., 1989). The functions of Müller cells were studied by employing specific toxins. DL-alpha aminoadipate is one such specific toxin that completely damaged Müller cells leading to a breakdown of histogenesis when used at high concentrations (Reinicke and Layer, 1992; Germer et al., 1997). Intra-vitreal injection of DL-alpha aminoadipate caused severe cytotoxic effects of Müller cells in adult rat (Pederson and Lund Karlson, 1979). Recent studies have shown that at a lower concentration, DL-alpha aminoadipate induced progenitor properties of Müller cells leading them to re-enter cell proliferation in adult mice (Takeda et al., 2008).

Alpha-amino-adipic acid is an intermediate amino acid in the breakdown of lysine in mammals. It is metabolised to alpha-ketoadipic acid and ultimately to acetoacetyl-CoA (Milton and Brown, 1980). Apart from being an intermediate in lysine metabolism, it is a six-carbon chemical analog of the excitatory amino acid L-Glutamate. We used a lower concentration of DL-alpha aminoadipic acid to the cultures of chick retinospheroids in order to follow the possible effects of the toxin on Müller glial cells, and indirectly onto neurons, up from the beginning of development of spheres.

Materials and Methods

Cell preparation and culture

Whole eyes from E6 chicken embryos (White Leghorn) were isolated, washed with Hanks´ medium and dissected free of connective tissue. The lens, the vitreous body and the pigment epithelium were removed. The retinas were collected in F12 medium on ice. For dissociation, the retinas were treated with 1mg/ml trypsin for 10 min at room temperature. The tissues were then rinsed in F12 medium and gently dissociated into single cells with a fire polished Pasteur pipette in the presence of 0.05 mg/ml DNase. After a threefold wash in F12 medium, the cells were resuspended in culture medium (10% fetal calf serum (FCS), 2% chicken serum, 1% L-glutamine, 0.1% penicillin/streptomycin, 0.02 mg/ml gentamycin in DMEM medium). Cells were suspended in such a manner that each plastic dish of 3.5 cm contained two eyes in 2 ml of culture medium. The cells were reaggregated by rotation on a gyratory shaker (72 rpm, 37°C, 95% air/ 5% CO_2). The medium was changed once in two days.

Supplements

The cultures were maintained in two different sets. The first set of culture served as the control. The next set of culture was treated with 0.4 mM DL-alpha-aminoadipate (AAA), a Müller glia toxin (final concentration in 2 ml culture media). The retinospheres were cultured for a period of 10 days.

Fixation of spheroids for cryosectioning

For preparation of frozen sections, the aggregates were first washed thrice with phosphate buffered saline (PBS) in order to remove the utilized medium. Then the aggregates were fixed in 4% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed again three times in PBS for 10 min each and embedded in a solution of 25% sucrose in PBS and sectioned at 10 to 14 micrometer thickness on a cryostat. The sections were dried and stored at -20°C for further immunostainings. At parallel times, spheroids were collected, washed thrice with PBS and centrifuged at 10,000 rpm for 5 min. The supernatant was completely removed and the spheres were stored at -20°C degrees for further homogenate preparation for molecular biology techniques.

Immunohistochemistry

The frozen sections were pre-warmed at 37°C. The sections were marked around with a liquid blocker so that the staining reagents could stay within the areas marked. The slides were pre-treated with blocking solution (1% bovine serum albumin, 1% Triton X-100 in PBS) for 30-60 min. The blocked sections were treated with 100 microlitres of primary antibody of interest (primary antibody diluted in blocking solution). The slides were incubated either overnight at 4°C or for 70 min at room temperature. Then the slides were washed thrice with 1x PBS. The slides were then treated with 100microlitres of secondary antibody (secondary antibody diluted in blocking solution) for 60 min at dark. The slides were again washed thrice with 1x PBS and 100 microlitres of DAPI added and incubated for 3 min at dark. The slides were finally washed thrice with 1x PBS, rinsed in distilled water, dried on a warm plate and covered with gelatine. The details of antibodies used are as follows: Glutamine synthetase (1:200); AChE (1:200); ChAT (1:1000); Pax6 (1:500); CERN906 (1:1000); CERN901 (1:1000); Axonin1 (1:500); F11 (1:20); Vimentin (1:500); Cy3 (1:200); Cy2 (1:200).

TUNEL histochemistry

Cellular apoptosis was analyzed by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling). The staining was performed as per the manufacturer's protocol (Roche). In brief, the frozen cryosections were dried and incubated in permeabilising solution (0.2% Triton-X100 in PBS) for 5 min at room temperature, washed twice with 1X PBS and incubated in equilibration buffer for 5-10 min at room temperature. The sections were treated with the TUNEL incubation buffer (equilibration buffer, nucleotide mix and TdT enzyme) and incubated at 37°C for 1 hr. The reaction was stopped with 2XSSC buffer supplied in the kit and washed thrice in 1X PBS. Finally the sections were stained with DAPI for 3 min, washed, dried and covered with gelatine. For double staining, the final step of DAPI was performed after treating the sections with corresponding antibody staining.

Molecular biology studies

RNA Isolation

The spheres were treated with TRI Reagent (Sigma). RNA was isolated according to the protocol supplied by the manufacturer. In brief, the spheres were homogenized in the presence of TRI reagent by sonication. The homogenate was treated with chloroform to remove protein. RNA was isolated by isopropanol, washed with 75% ethanol and dissolved in DEPC water. Isolated RNA was treated with DNase to remove any DNA if present and stored at -20°C.

Reverse Transcription

cDNA (complementary DNA) was synthesized using AMV Reverse Transcriptase (Promega). The synthesis was according to the manufacturer's protocol with minor changes. In brief, the RNA was reverse transcribed to cDNA in the presence of 25 mM $MgCl_2$, 10 mM dNTP mix, Random or Oligo (dT) primer, AMV Reverse Transcriptase enzyme and RNasin, Ribonuclease inhibitor. Reverse transcription was carried out by incubating the mixture at 42°C for 30 min and inhibition of the enzyme was carried out at 95°C for 5 min and cooled at 0-5°C for 5 min.

Polymerase Chain Reaction

PCR was carried out by amplifying the first-strand cDNA in the presence of 25 mM $MgCl_2$, 10 mM dNTP mixture, 50picomoles each of both upstream and downstream primers, 2.5 Units Taq Polymerase enzyme (BioLabs), taq polymerase buffer and nuclease free water. Primers were synthesized by using Primer3 web based program and ordered from Euroffins Biotech, Munich, Germany. The details of the primers used for PCR are as follows: GAPDH sense: *CCTCTCTGGCAAAGTCCAAG*, antisense: *TGGCTGTCACCATTGAAGTC*; AChE sense: *GGTCGAGGGTTCCTATTTCC*, antisense: *ATCCACGAAGGCCACAATAG*; Rhodopsin sense: *ACTCCGGACGCCTCTAAACT*, antisense: *TCCTTCACAGTGCAGACCAG*; R/G op*sin sense: CAGAAGCAGTAGGCCACGAT*, antisense: *ATCAACCAGATCTCGGGGTA*.

Microscopy and Imaging

Immunostained *in vivo*, explant and spheroid sections were subjected to fluorescent microscopy (Axiophot, Carl Zeiss, Germany) which was connected to an automated digital camera (Axiocam). Imaging was done for each fluorescent channel separately and processed through extended focus imager, and all the pictures from different channels were combined as overlays and stored in zvi image format by Axiovision Basis software programme.

Results

Development of retinospheroids

Dissociated retinal cells from E6 chick embryo reaggregated under constant rotation cultures to form histotypical structures resembling an in vivo retina. Such type of structures was achieved within 6 days of culture. Initially the single cells closely reaggregated without any remarkable structures around 2 days of culture. After 4 days, cells began to sort out resulting in the formation of fluid filled cell free spaces which were either recognized as rosettes or plexiform-like areas based on the cell types that surrounded the spaces. Photoreceptors normally aggregated together to form the rosettes, while the inner retinal cells sorted to form the inner plexiform-like areas. A complete histotypical structure containing all the retinal layers was achieved within 10 days of culture (Fig. 1).

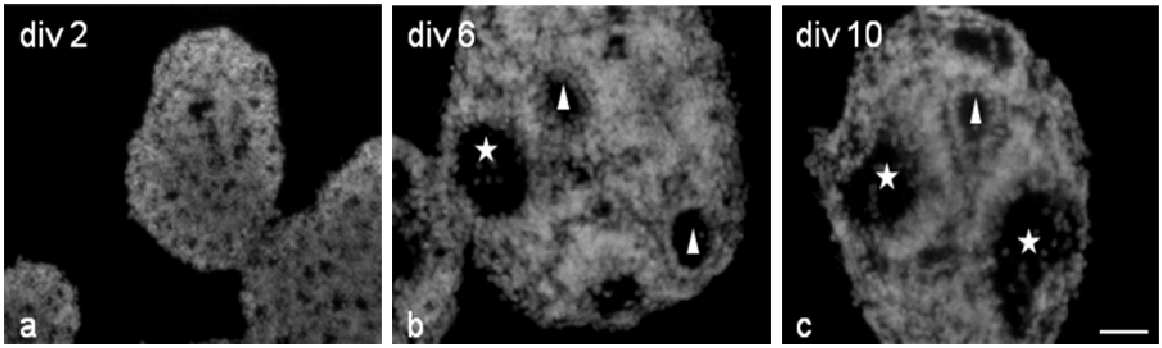

Fig.1: Development of dissociated retinal cells in vitro. Dissociated retinal cells from E6 chick embryos reaggregate to form histotypical structures called retinospheroids. Sequential development of the histotypical structures is shown (a-c). At 2 days in vitro (div), single cells reaggregated to form a sphere which looked like a mere ball filled with cells (a). At 6 div, cells undergo sorting, a process during which "like cells" tend to assemble together. This resulted in the formation of cell free spaces which can be recognized either as inner plexiform layer (IPL) (star in b) or a photoreceptor rosette (triangle), based on the cell types surrounding the area. Similar structures (IPL – star and rosette – triangle) are found even after 10 days of culture (c) making the system advantageous to follow developmental and toxicological assessments. Scale = 50 µm.

DL-AAA alters the circularity of spheres

During normal development of spheres, until 4 days of culture, the spheres remained circular when there was not much sorting of cells. After 4 days, the cells began to sort-out dramatically which led to the alteration in the shape of spheres. The spheres became irregular in shape, especially due to the bulging out of some areas where IPL-like areas formed which were either cell free or contained very few cells. After 8 days, most of the bulging areas disappeared and the

spheres became circular. A 0.4 mM concentration of the glial toxin DL-AAA altered the shape of the spheres to a large extent. Under the treatment of AAA, even after 10 days of culture the spheres never achieved circularity and remained irregular (Fig. 2).

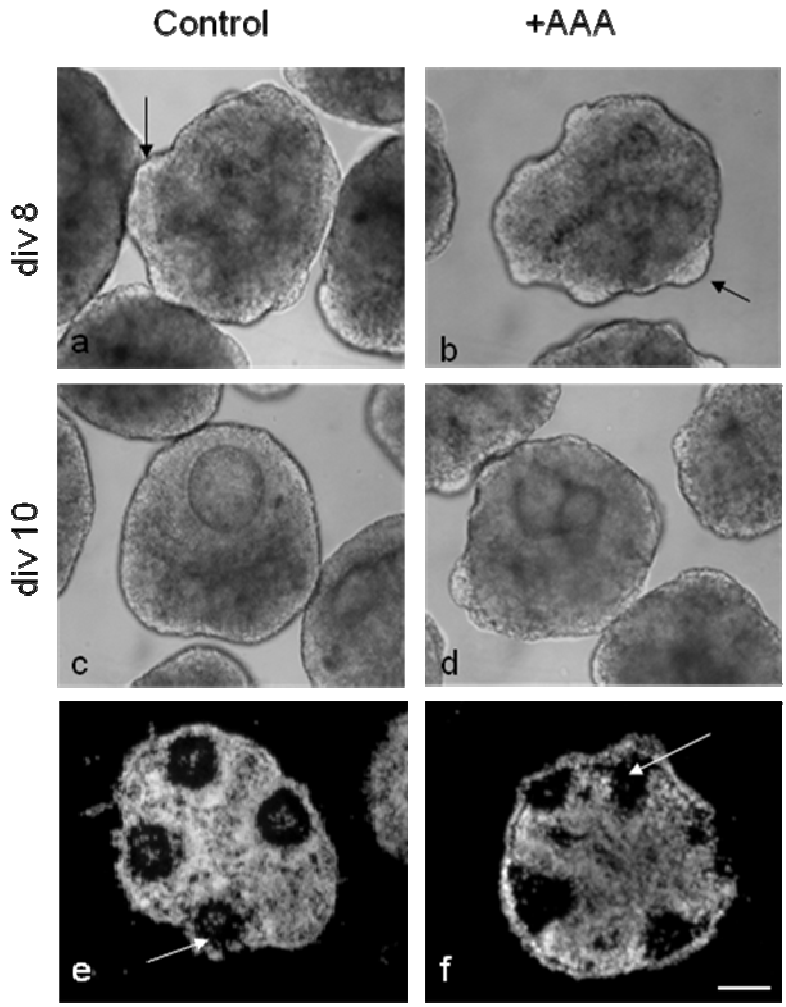

Fig.2: DL-AAA affects the spheres morphologically. Light microscopic pictures of 8 and 10 div spheres (a-d). Control spheres which are untreated, have irregular shape due to cellular movements and formation of IPL-like areas (bulging out of areas shown in arrows in a, b) during development and later achieve near-circular morphology (c). Treatment with 0.4 mM DL-AAA rendered the spheres irregular on both culture days (b, d). Sections of div 10 control and AAA-treated spheres stained for DAPI (e, f). Note that the bulging areas in control and AAA-treated spheres corresponding to the IPL-like areas (arrows in e, f). Scale bar = 50 µm.

Effect of DL-AAA on Müller glial cells

In non-treated control spheres, by 8 days of culture, differentiated Müller cells exhibited long radially organized processes throughout the retinal spheres, as revealed by glutamine synthetase immunostaining (Fig. 3a). The long processes were seen spreading deep into the IPL-like areas. Under DL-AAA treat-

ment, the processes were shortened, especially in the IPL-like areas (Fig. 3b). It is well known that DL-AAA is a toxin for Müller cells with high specificity (Olney et al., 1971). F11, a neural cell recognition molecule is expressed on Müller cells. The intermediate filament vimentin along with F11 was also used as a marker to observe the effects of DL-AAA. In control spheres, both F11 and vimentin were localized in the long radial process of the Müller cells (Fig. 4a-e). But interestingly, in DL-AAA treated spheres, unlike glutamine synthetase immunostaining, which was absent in the processes of the cells, F11 and vimentin were still observed in the latter (Fig. 4f-j), suggesting a non-toxic effect of DL-AAA.

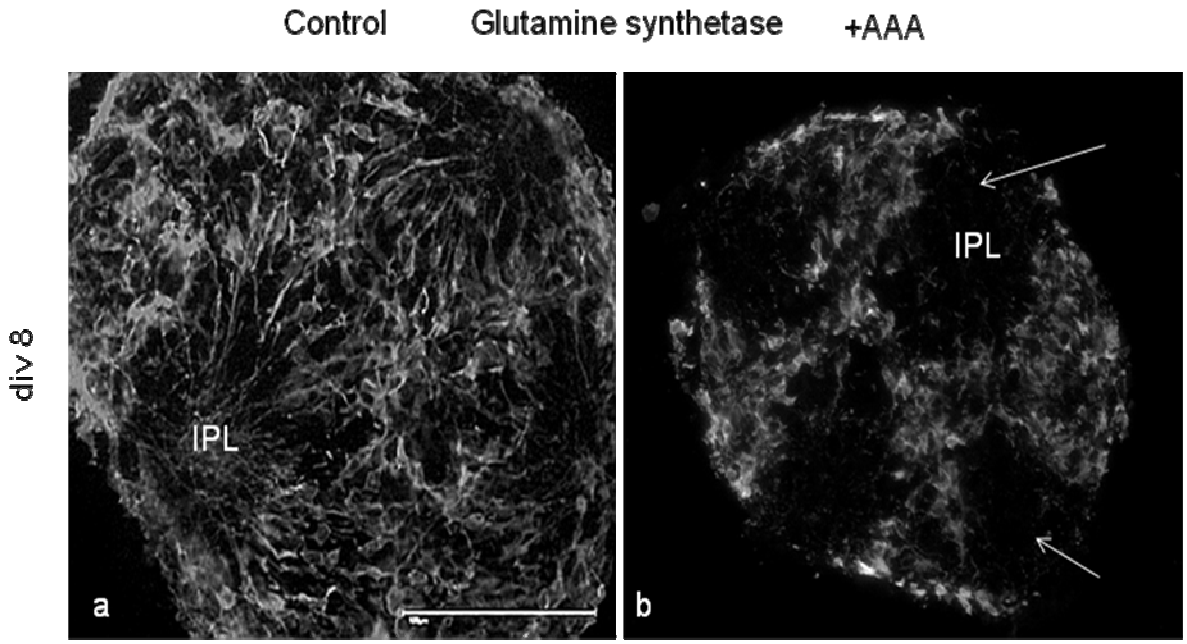

Fig. 3: The pictures represent immunostaining for glutamine synthetase expression of Müller glial cells. In 8 div control spheres, long radial processes of Müller cells extending deep into the IPL-like areas could be noticed (a). Under DL-AAA treatment, the radial processes are severely disturbed, where the IPL areas are totally free of staining (arrows in b).
Abbrev: IPL - inner plexiform layer. Scale bar = 50 µm. Picture (a) was prepared by Gesine Bachmann.

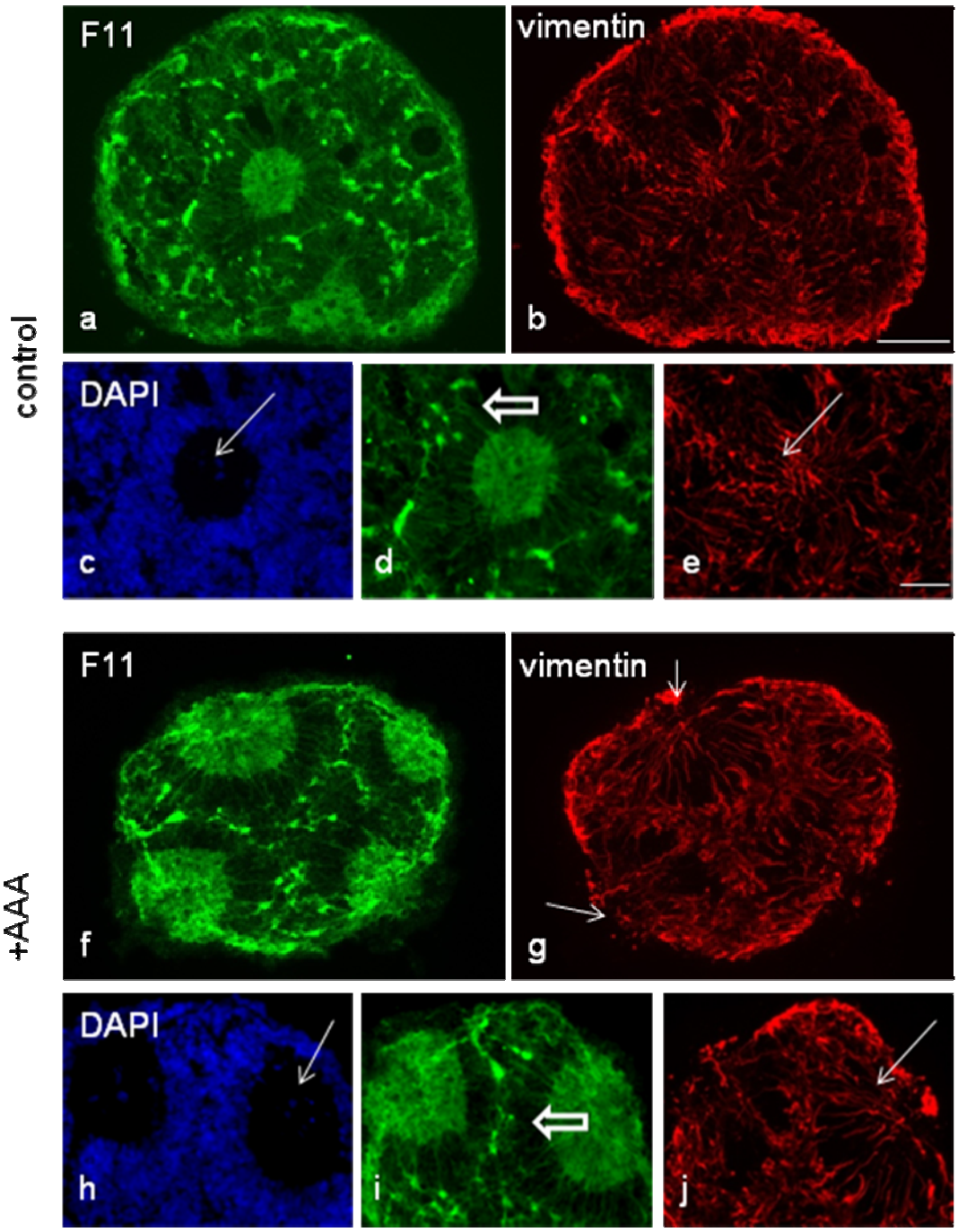

Fig. 4: F11 (green) and vimentin (red) immunostaining in control and DL-AAA spheres. The pictures show div 8 spheres. (a): The cell recognition molecule F11 is found in Müller cells and is located on radially arranged Müller glia processes which reach deep into the IPL. The intermediate filament protein vimentin is localized in the processes of Müller cells as well (b). The corresponding IPL-like area is depicted by DAPI-free (blue) areas in c (arrow). (d and e) are higher magnification pictures of the IPL-like area and the radial processes of Müller glial cells stained for F11 (block arrow in d) and vimentin (arrow in e). Section of an eight day-old sphere treated with AAA and stained for F11 and vimentin is shown in f-j. Radial processes of glial cells are still observed. Unlike glutamine synthetase, vimentin immunostaining is still present in the processes in the IPL, but the processes are not connected in the outer side of the IPL (arrows in g). IPL is shown by DAPI staining (h). Higher magnification of the IPL where the processes stained for F11 (block arrow in i) and vimentin (arrow in j) are shown. Scale bar = 50 µm (a, b, f and g); 20 µm (c-e, h-j).

The toxic effect of DL-AAA was studied by TUNEL histochemistry. High concentration of DL-AAA is expected to exert drastic effects on differentiated Müller glial cells rather than progenitors and other neuronal cells. In spheres, differentiation of Müller cells was achieved within 8 days of culture, revealed at least by glutamine synthetase immunostaining, as this enzyme is a well accepted marker of mature Müller cells.

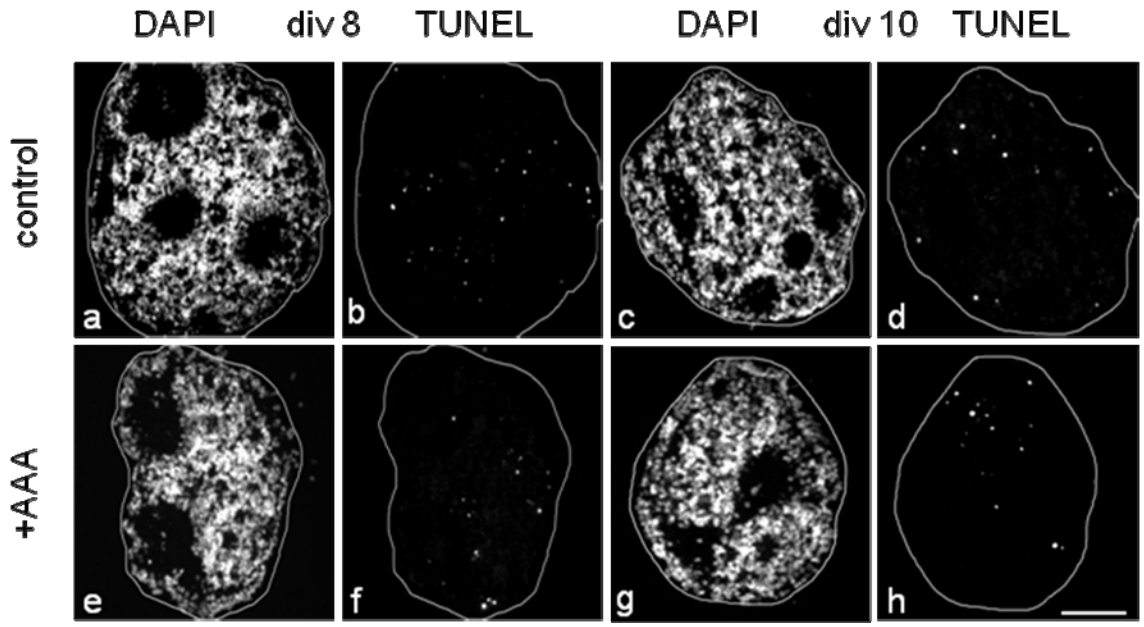

Fig. 5: TUNEL histochemistry for cellular apoptosis in control and toxin treated spheres. Even under normal conditions (control), a few cells undergo apoptosis as the process of development proceeds. Sections of 8 and 10 div spheres subjected to TUNEL assay showed a very few cells undergoing apoptosis (a-d; a, c are corresponding DAPI pictures for the same sections stained for TUNEL in b, d). Treatment of spheres with DL-AAA (e-h) till 10 div did not show any significant number of cells undergoing apoptosis. Scale bar = 50 µm.

Spheres of 8 and 10 days culture were subjected to TUNEL staining. Interestingly, the results indicated that the apoptotic levels in spheres treated with DL-AAA were almost equal to those of control spheres, suggesting that the effect was not associated with Müller cell death when the toxin was present from the beginning of the culture (Fig. 5). Even when the spheres which were allowed to develop at least till 8 div, a stage when the Müller cells are expected to differentiate in vitro, and then treated with the same concentration of DL-AAA, most of the

Müller cells survived except for a few cells which were associated with apoptosis as revealed by TUNEL staining (Fig. 6).

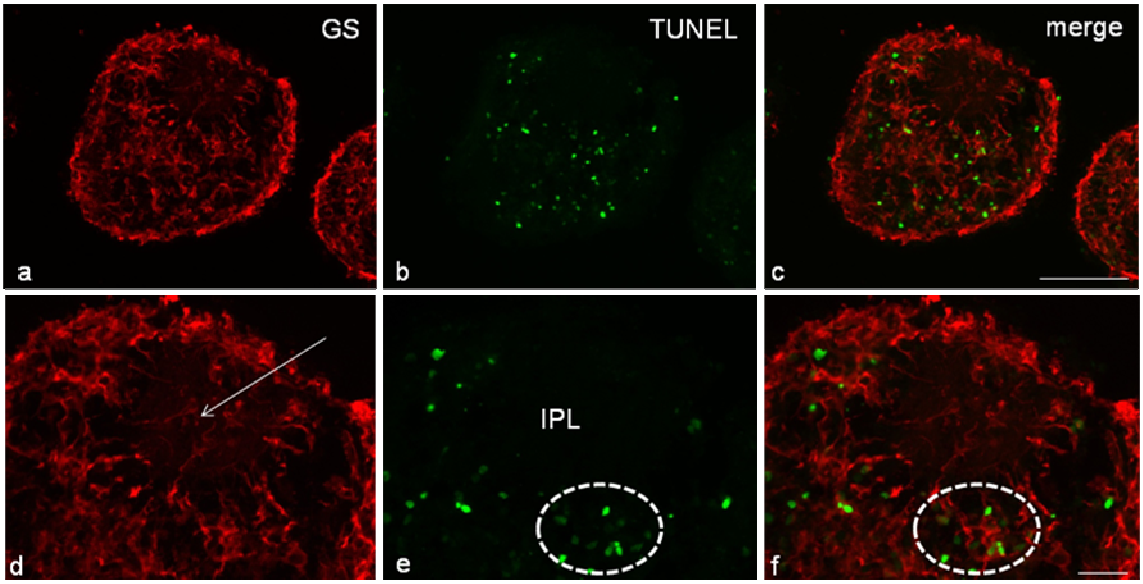

Fig. 6: Pictures of 12 div spheres which were treated by 0.4 mM DL-AAA on the eighth day of culture. (a) shows glutamine synthetase staining. Note the radial processes had shortened in the IPL like areas (a higher magnification is shown in d, IPL; arrow). The same section labeled for apoptosis by TUNEL is shown in b. Note a few more cells are associated with cell death when compared to cultures which had DL-AAA from the beginning of the culture period (Fig. 4). A higher magnification is shown in e. Merge channels are shown in c and f. Co-localization of TUNEL+ in Müller cells are shown in ellipsoids in e and f.
Abbrev: IPL – inner plexiform layer. Scale bar = 50 µm (a-c); 20 µm (d-f).

IPL subband formation is disturbed by DL-AAA treatment

As mentioned above (Fig. 1), after 4 days of culture, the cells in spheres rapidly sort out and result in the formation of cell free spaces which were recognized as IPL-like areas, based on cell types which form these spaces. Similar to in vivo developmental stages (discussed in chapter I), migration of cholinergic amacrine cells towards the ganglion cell layer (GCL), forming the displaced amacrine cell layer was observed in the spheres as well. These cells are termed type II cholinergic amacrine or displaced starburst amacrine cells (dSACs). Conventional, or type I cholinergic amacrine cells were found at the outer border of the IPL-like areas, as revealed by choline acetyltransferase (ChAT) and acetyl cholinesterase (AChE) immunostaining. The processes from both the cell types reached the cell-free space between them and met each other which resulted in the formation of a continuous band. This continuous subband was a prominent cholinergic subband (Fig 7a). In spheres treated with DL-AAA, the formation of an

IPL subband was severely disturbed (Fig 7b) suggesting that Müller cells play major roles in establishing and/or maintaining the IPL subbands.

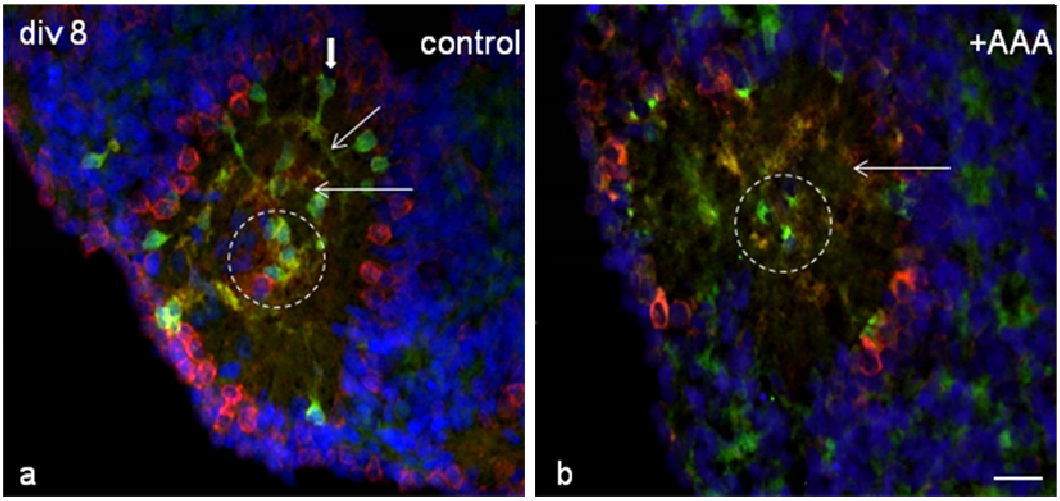

Fig. 7: Cholinergic subband formation. An eight day-old control sphere exhibiting an IPL and the cholinergic subbands in vitro, shown by immunostaining with AChE (red) and ChAT (green). Cell nuclei are shown with DAPI (blue). Similar to in vivo organization, the type I cholinergic amacrine cells are found at the border of the IPL/INL (a type I cell marked with a block arrow in picture a). The type II cells which had migrated into the IPL are shown in broken circle. The two cholinergic subbands could be noticed in the IPL (arrows). Under DL-AAA treatment, the organization of both the type I and type II cholinergic cells is disturbed (b). Though a few type II cells still migrated into the IPL (broken circle), the cholinergic subbands are severely disrupted (arrow). Scale bar = 20 µm.

DL-AAA increases the inner retinal cells

The disturbance in the IPL subbands under AAA treatment could be attributed to the effect of the toxin on the Müller cell processes but surprisingly there was an increase in the inner retinal cell types as revealed by different amacrine cell markers. Staining by 3D10 antibodies against AChE showed an increase in the number of cells expressing the protein in the inner nuclear layer (INL). In vivo, during early retinal development, more number of cells expressed AChE in the INL almost occupying half of the layer and later most of the cells down-regulate the enzyme. In an adult chick retina, the $AChE^+$ cells appeared in just two or three rows in the INL (own observation, discussed in chapter IV). In a similar fashion, more cells began to express the enzyme around the first 6 days of culture (not shown) and then onwards the number of cells expressing AChE decreased constantly (Fig. 8Aa-d). Under AAA treatment, the number of $AChE^+$ cells remained higher even at 10 days of culture (Fig. 8Ae-h). Further analysis by RT-

PCR also showed a rise in the AChE mRNA synthesis under the toxin treatment (Fig. 8B).

Pax6 was used as a marker of inner retinal cells. It is a transcriptional factor expressed very early in the proliferating cells, but later is retained only by the different type of amacrine cells in the INL and the GCL. As expected, control spheres showed an initial rise in Pax6 expression during early culture periods (not shown), and was later retained by a few layers of cells surrounding the IPL spaces (Fig. 9b, d).

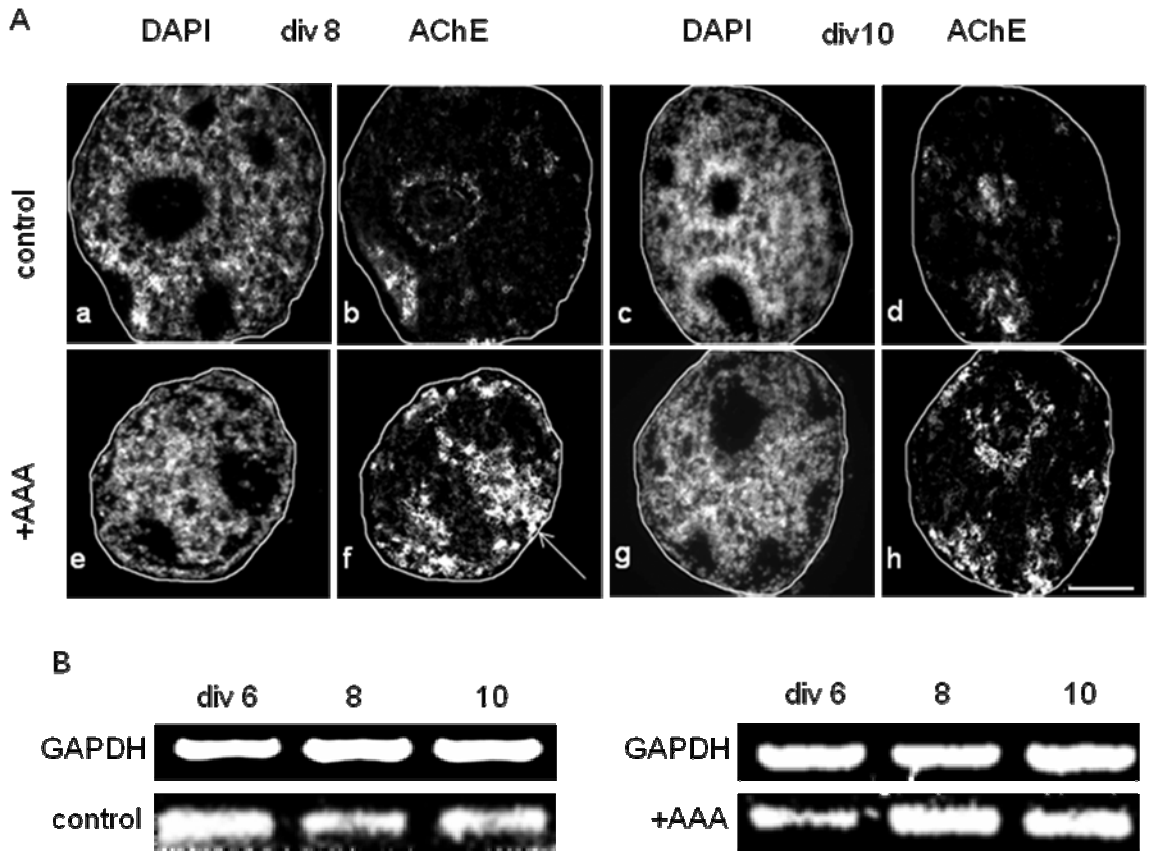

Fig.8: Expression of AChE. A: Immunostaining of spheroid sections for AChE by using 3D10 antibodies. Control spheres of div 8 and 10 are shown in (a-d). The DAPI stained sections showing the whole spheres of div 8 and 10 (a and c). Note that AChE is expressed in cells around the IPL (arrows) and also in the subband (b). By 10 div, the cells around the IPL had already reduced the expression and the protein is found in the IPL (d). DAPI stained sections of spheres treated with AAA are shown in (e and g). Note a drastic increase in the protein expression by 8 div (f, arrow). By 10 div, AChE expression is reduced but comparatively higher than control (h). B: mRNA expression of AChE by RT-PCR. Lane 1 on left shows expression of GAPDH at 6, 8 and 10 div which served as control for gene expression. Lane 2 shows AChE expression in control spheres at the corresponding culture days. Note that the expression gradually decreases by time. Lane 1 on right shows expression of GAPDH in DL-AAA treated spheres. Lane 2 shows the protein expression under AAA treatment. Though the expression is low at div 6, div 8 and 10 spheres have more expression than the control spheres. Scale bar = 50 µm.

Under AAA treatment, more cells retained Pax6 at the same culture periods compared with the control (Fig. 9f, h).

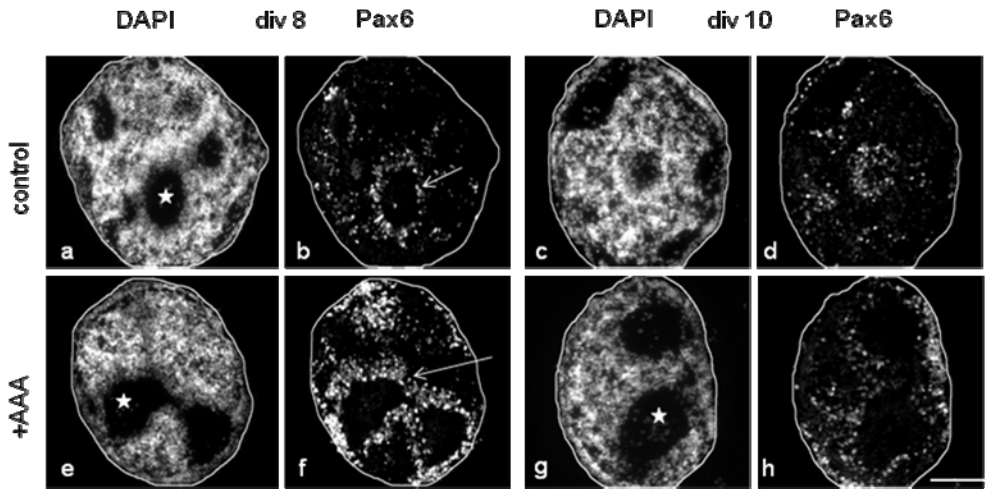

Fig. 9: Immunostaining of Pax6 as a marker for inner retinal cells (amacrine cells). (a-d) show pictures of div 8 and 10 control spheres whose DAPI staining is shown in (a and c; IPL is marked by star in a). Note $Pax6^+$ cells around the IPL (arrow in b). By 10 div, the number of Pax6 cells is reduced (d). Spheres treated with DL-AAA are shown in e-h. IPL-like structures are bigger in treated spheres than control (stars in e and g). Note a drastic increase in the numbers of $Pax6^+$ cells (arrow in f) at both culture days (f and h). Scale bar = 50 µm.

Axonin1, an adhesive protein specific for amacrine cells was used as an additional marker in order to see whether the effect reflected in the synthesis of such proteins.

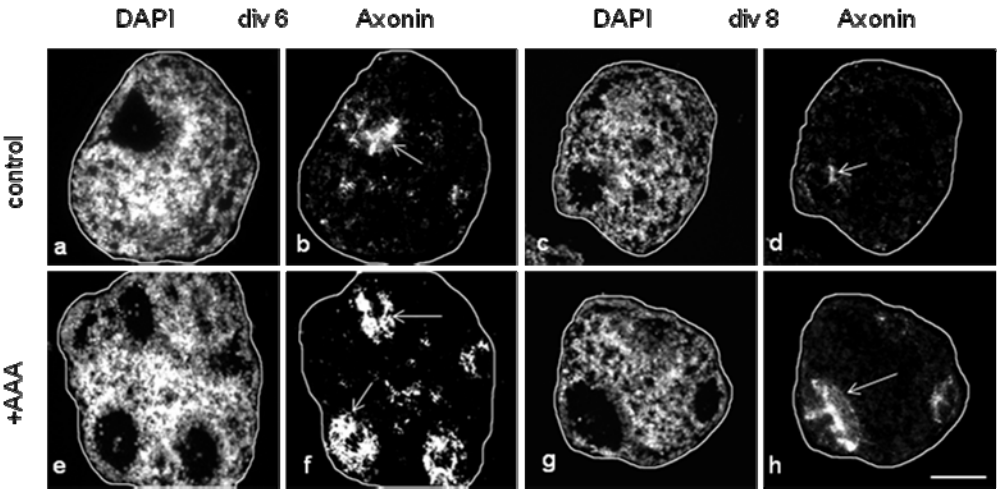

Fig. 10: Axonin1-immunostaining in the inner retina. Control spheres of div 6 and 8 are shown in (a-d). Cell nuclei are shown by DAPI staining. Expression of axonin1 is observed only in the IPL (arrow in b). By 8 div, expression of the protein is very low in the control spheres (d). In AAA treated spheres, the number of IPL areas is more than control as observed by DAPI (e). All the IPL areas are filled with amacrine cell processes stained for axonin1 (arrows in f). Interestingly, even at div 8, the expression of the protein is strong (arrow in h). Scale bar = 50 µm.

The protein is localized only in the cell processes, but not in the cell soma. Control spheres expressed the protein at 4 days in culture (not shown) and down-regulated it by 8 days (Fig. 10a-d). Interestingly, spheres treated with AAA showed intense immunoreactivity in the IPL even at 8 days of culture (Fig. 10e-h).

DL-AAA reduces photoreceptor differentiation

Normally, photoreceptor differentiation in spheres began after 4 days in culture. But the expression of visinin, a marker for photoreceptor progenitors was observed before 4 days (not shown).

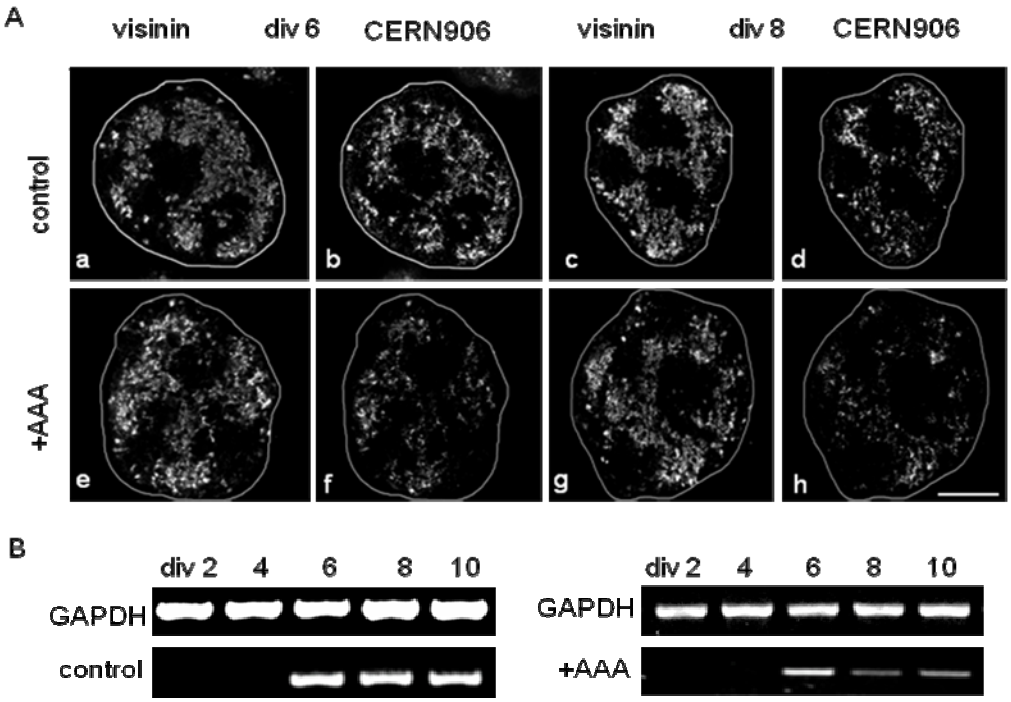

Fig. 11: Differentiation of cone photoreceptors in control and DL-AAA treated spheres. Immuno-stainings of visinin and CERN906 in div 6 and 8 control spheres are shown (a-d). a, c are sections stained for visinin at div 6 and 8, while b, d are corresponding sections stained for CERN906. Note the photoreceptor cells are diffusely scattered throughout the spheres. Under DL-AAA treatment, expression of visinin is almost equal to that of control spheres (e, g). But expression of cone opsin was reduced when compared with that of the controls (f, h). B: RT-PCR for the expression of cone opsin. Lane 1 on left shows the expression of GAPDH from div 2 through div 10. Lane 2 shows the expression of cone opsin in control spheres. Lane 1 on right shows GAPDH for spheres treated with DL-AAA from div 2 to 10. Lane 2 shows the expression of cone opsins in treated spheres. Note that the expression of cone opsin mRNA begins only after 4 div. Note dramatic decrease in the mRNA expression when compared to that of control. Scale bar = 50 µm.

CERN 901 and CERN 906 antibodies were used as differentiation markers for differentiating rod and cone photoreceptors. Expression of visinin and CERN

906 in control spheres at days 6 and 8 is shown in Fig. 11A, a-d. Under AAA treatment, the expression of visinin was not affected but the differentiation of photoreceptors was reduced as revealed by the immunostaining (Fig. 11A, e-h). Further analysis by RT-PCR also showed the corresponding down-regulation in the expression of rhodopsin mRNA after AAA treatment, respectively (Fig. 11B). Similar results were obtained for differentiation of cone opsins under the toxin treatment (Fig. 12A, B).

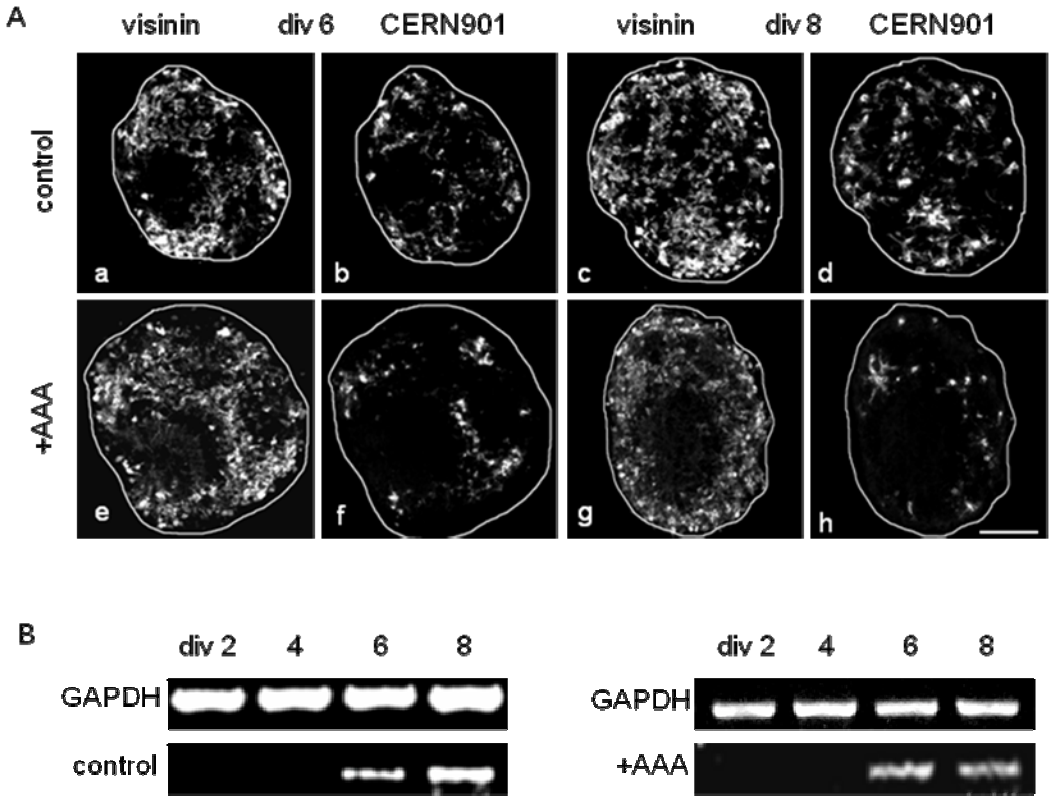

Fig. 12: Differentiation of rhodopsin in control and DL-AAA treated spheres. (A): Immunostaining of visinin and CERN901. (a, c) show the expression of visinin in div 6 and 8 control spheres. (b, d) show expression of CERN901 in corresponding sections. Note that similar to cones, the rod opsins are also diffusely scattered throughout the spheres. Under AAA treatment, the expression of visinin as reported earlier, was normal like that of controls, while the expression of CERN901 is remarkably reduced. (B): RT-PCR for the expression of rhodopsin. Lane 1 on left shows the expression of GAPDH from div 2 through div 8. Lane 2 shows the expression of rhodopsin in control spheres. Lane 1 on right shows GAPDH for spheres treated with DL-AAA from div 2 to 8. Lane 2 shows the expression of rhodopsin in treated spheres. Note that the expression of cone opsin mRNA begins only after 4 div. Note the decrease in the mRNA expression when compared to that of control spheres. Scale bar = 50 µm.

Discussion

In vivo studies are quite indispensable as they only can give a proof of any biological process, be it a biochemical reaction or complex tissue formation or normal developmental processes that occur during any tissue or organ growth. However, experiments related to the biological functions are difficult to conduct. It is often a requirement of in vitro systems that mimics an in vivo system to overcome experimental difficulties that need to be carried out in order to understand the functions of certain cell types, or proteins involved in the development and organization of complex tissues. The retinospheroid technology is one such powerful in vitro technique that had been developed since decades, especially for development studies of retina. This system is based upon the capacity of chicken retinal cells that are enzymatically dissociated to single cells and allowed to reaggregate, proliferate, differentiate and re-organize themselves into histotypic structures (Adler, 1973; Akagawa et al., 1987; further reviewed by Willbold and Layer, 1998). Compared to in vitro systems like monolayer or slice cultures, retinospheroids offer a number of advantages: the first being a three dimensional system in which growth of tissues is not restricted to two dimensions. The next important advantage being the ability of single cells to build up to complex structures which allows investigating the developmental processes step by step. Retinospheres developed from E6 chick embryos contain the neuronal cells and the Müller glial cells like that of an in vivo retina. Development of plexiform layers has also made this system a more powerful in vitro technique.

Though the retinal neurons and Müller glial cells share common progenitors (Turner and Cepko, 1987), the latter is known to show tremendous plasticity during the development of the retina (Burke and Smith, 1981; Reichenbach and Robinson, 1995a). Müller cell precursors supposedly are present from early stages of retinal development, and due to their remarkable plasticity during maturation, their morphological shape, biochemical equipment and functions change continuously (Willbold and Layer, 1998).

Role of Müller glial cells in laminar organization

One of the most astonishing features of the retina is its columnar organization, like that of other brain parts (Rakic, 1972; Leber et al., 1990). In these brain parts, radial glial cells act as guiding factors for migrating neurons to find their

destination (Hatten 1990, 1993). Similarly, the Müller glial cells were suggested to be the guiding factors of migrating neurons in the retina (Morest, 1970; Snow and Robson, 1995). During early developmental stages in spheres, INL-homologous areas could be seen where all retinal neurons including photoreceptors, horizontal, bipolar and amacrine cells are present. Müller glial cells could also be observed very early in these INL-like areas which are non-organized. Then the IPL-like areas which are mainly composed of cellular processes begin to form which are also filled with long radial processes of the Müller glial cells. Unlike that of an in vivo retina, an ILM is not formed in the retinospheroids and the processes of Müller cells end freely in the IPL (Wolburg et al., 1991). To observe how Müller cells might achieve these functions, we used DL-alpha aminoadipate, a potential Müller glial toxin (Olney et al., 1971; Kato et al., 1990, 1996; Willbold et al., 1995).

DL-AAA exerts sub-toxic effects when present before maturation of Müller cells

Unlike previous studies where high concentration of DL-AAA caused severe destructions to the whole spheres, we used a lower concentration (0.4 mM) of DL-AAA in order to target only the Müller glial cells. Also different from earlier studies where the toxin was introduced at a late stage of development when already the Müller cells were differentiated, we treated the spheres from the beginning of the culture. However, the Müller cells still developed even in the presence of the toxin before their differentiation. But noticeably, their long radial processes were not associated with glutamine synthetase immunostaining (Fig. 3b). But surprisingly, as observed by vimentin immunostaining, long radial processes could still be noticed in the IPL-like areas of treated spheres. Vimentin is an intermediate filament protein expressed by early Müller glial cells, and is later retained by the cells even after maturation. Further, F11 was also used as a marker in order to observe the fate of Müller cells in the presence of the toxin. F11 is found to be involved in cell contact-dependent modulation of neuronal differentiation, neurite outgrowth and fasciculation (Brümmendorf and Rathjen, 1995). In retina in vivo, F11 was observed in the inner and outer plexiform layer (Layer and Willbold, 1989, 1994; Rathjen et al., 1991). Willbold et al. showed that the protein was expressed in radially organized structures in stratospheres (another 3D reti-

nal model of reaggregate system), which were identical to Müller cells (Willbold et al., 1997a). Interestingly, the processes immunostained for F11 were parallel like those of vimentin even in toxin-treated spheres (Fig. 4), suggesting that cellular interactions were not completely lost. Also TUNEL assays suggested that the Müller cells were not associated with apoptosis, supporting the notion that DL-AAA exerts sub-toxic effects when present at a lower concentration from the beginning of the culture. Based on our observations, we could interpret that DL-AAA might delay the maturation of Müller cells rather than destroying them when present in the culture before glial differentiation.

DL-AAA disrupts the cholinergic stratification, but increases the inner retinal cells

Cholinergic stratification in the IPL begins around E9 in chick retina in vivo (Reiss et al., 1996). But the differentiation of Müller glial cells based on the expression of glutamine synthetase is expected to occur much later. Then, how could Müller cells play a role in the stratification of the IPL? Since undifferentiated Müller glial cells are present from the beginning of the culture, they might have important functional implications during the formation of the IPL and its laminar stratification, as the glial cells might guide the migration of cells involved in the development of the IPL and also their neurites. A disturbed pattern in the migration of displaced amacrine cells was observed in a Golgi study (Prada et al., 1989). In spheres treated with DL-AAA, the displaced amacrine cells migrated to the IPL but were not arranged regularly as compared to that of control spheres. However, the cholinergic stratification was not formed, as revealed by the cholinergic markers ChAT and AChE. On the other hand, the numbers of inner retinal cells were increased, as revealed by different amacrine cell markers including AChE, Pax6, and axonin1. In vivo, AChE is expressed by amacrine cells both in INL and GCL. As development proceeds, the expression of the enzyme is restricted only to one or two rows of cells in the INL and GCL (discussed in chapters I and IV). Similarly, the expression of the transcription factor Pax6 increases during early development stages but later down-regulated and was retained only by a few amacrine cells in the INL. Axonin1 is a cell adhesive protein expressed in amacrine cells from E8 onwards in chick retina (Drenhaus et al., 2004). During development, the protein is localized in two or three subbands in the IPL. By E15

onwards the expression was down-regulated both in cell bodies and in the stratified bands in the IPL. In vitro, a similar pattern of expression occurs where the expression is observed before day 4 and is down-regulated by day 8 of culture. It is to be remembered that the development processes are much more advanced in vitro (Frohns et al., 2009; Thangaraj et al., 2011). Surprisingly, under the treatment of DL-AAA, all the markers mentioned above were found increased. Such observations lead to open questions as to whether DL-AAA acts only on Müller glial cells, or whether it could act through unknown mechanisms also onto neurons, or does it exert such effects indirectly through Müller cells, etc.? Though earlier reports suggested that DL-AAA is a specific toxin for Müller glial cells (Bonaventure et al., 1981; Kato et al., 1993, 1996; Linser and Moscona, 1981; Olney et al., 1971), our observations require a reconsideration of its mode of action and specificity.

DL-AAA hinders photoreceptor differentiation in vitro

Using the same gliotoxin, earlier reports suggested that Müller cells play major roles in the final maturation of photoreceptors (Rich et al., 1995; West et al., 2008). We used visinin as a marker for early photoreceptors and CERN901 and 906 to observe differentiating photoreceptors. Some of the studies reported the expression of visinin, a calcium-binding protein to be cone-specific (Yamagata et al., 1990). We observed that the protein was expressed as early as 2 days in vitro, when CERN901 and 906 were not expressed, suggesting that the cells were immature photoreceptors and later retained their expression even in differentiated cells. Under normal conditions (untreated), the photoreceptors began their differentiation after 4 days in vitro. In DL-AAA treated spheres, though the expression of the proteins began around the same culture period, the number of cells expressing the markers was very less. But there was not any difference in the number of cells expressing visinin in the treated spheres suggesting that the toxin hinders the differentiation of photoreceptors.

Does DL-AAA induce a cell type shift in vitro?

The observations so far in our studies raised the question whether DL-AAA induces an early cell type shift towards the inner retinal cells directly or indirectly. It is already known that Müller glial cells possess tremendous capacity of

plasticity. Treatment with bFGF and/or insulin led to Müller cell dedifferentiation, as the cells re-entered the proliferative stage and were destined to become neurons in chick retina (Fischer et al., 2002; Frohns et al., 2009). In mouse, DL-AAA was found to induce progenitor cell properties (Takeda et al., 2008). However, our study failed to observe any increased cell proliferation after the treatment of retinospheres with DL-AAA. So the treatment might induce an early cell type shift towards the inner retinal cells. Whether DL-AAA exerts such type of cell shift by acting by unknown mechanisms through neurons, or indirectly, through Müller glial cells are to be answered in the future with more molecular biology studies revealing any such shifts associated with differential expression of genes.

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General Discussion

The nervous system (NS) of vertebrates is divided into a central and a peripheral part. The central nervous system of most vertebrates comprises of the brain, spinal cord and retina. In these parts, the neurons are the main cells that co-ordinate the actions of an organism and transmit signals between different body parts. These actions are attained by very complex neural pathways. Neurons communicate with other cells through their processes (axons and dendrites), which release neurotransmitters at their junctions, called synapses. Besides neurons, the nervous system contains glial cells which provide structural and metabolic support.

The development of neural systems occurs through defined steps, such as: 1. the birth of neurons, or neurogenesis, from stem cell precursors; 2. proliferation and migration of neurons to their final destinations; 3. differentiation and determination of immature neurons; and 4. axonal outgrowth, target finding and formation of synapses (Zigmond et al., 1999; Sanes et al., 2006). Since studying experimentally the development of the brain remains complicated, the retina as an outgrowth of the CNS provides a superior model tissue for analysis.

This thesis is grounded on long-standing work of Professor Layer and his colleagues, devoted to two major research areas: 1. the role of the cholinergic system in nervous system development, and 2. lamina and network formation during retinal development of the chick. My thesis contributes significantly to a better understanding of *both* topics, by having analyzed both *in vivo* and *in vitro* two major components of the cholinergic system (ChAT and AChE expression) during formation of the inner plexiform layer (IPL), as a primary step of retinal network formation. The IPL of any vertebrate retina is an incredibly complex, but highly ordered structure. Multiple sublaminae are arranged in parallel, each one fostering synapses of distinct cell types (see Introduction). As envisaged under “Aims of this study”, I could provide novel insights into the following topics:

- the cell lineage origin of cholinergic amacrine cells (SACs);
- the segregation of ganglion cells (GCs) from SACs;
- the time when SACs become cholinergic;
- that AChE appears earlier than ChAT, and thus raise the question what roles AChE might play in IPL development;

- that occurrence of SACs and their cholinergic IPL subbands is preceded by an AChE$^+$ subband in the IPL matrix;
- that SACs are required for stratification of the IPL;
- that MCs also contribute to stratification of the IPL;
- but: that GCs are not required for cholinergic subband formation.

This general discussion follows three lines, 1) what major knowledge provided the basis for this study, 2) what are my new findings, and 3) how do my findings extend our understanding of the mentioned two major topics? Finally, I will present hypotheses of how AChE might be involved in developmental cholinergic functioning. Such mechanisms might apply not only to IPL formation in the retina, but also to general neural network formation in nervous systems.

Cholinergic amacrine cells differentiate from a common pool of progenitors

I focused on the cholinergic amacrine cells as these cells differentiate very early following the ganglion cells. As the name indicates, the cholinergic amacrine cells which - based on their dendritic branching pattern - are also called starburst amacrines (SACs) (Famiglietti, 1983; Vaney, 1984; Masland and Tauchi, 1986), utilize acetylcholine (ACh) as a neurotransmitter. I used choline acetyltransferase (ChAT), the enzyme that synthesizes ACh and acetylcholinesterase (AChE), the enzyme that degrades ACh, as markers to follow the development of these cells. One or the other of these two enzymes has been used since decades to study the development of the cholinergic amacrine cells (Johnson and Epstein, 1986; Spira et al., 1987; Reiss et al., 1996). However, a detailed comparative study using both markers simultaneously was still missing. In my studies, I observed AChE at E4 in the presumptive ganglion cell layer (GCL) of the central retina, consistent with earlier reports (Layer, 1983). ChAT began to appear at E5 in some of the existing AChE$^+$ cells (chapter 1), while earlier studies reported the expression of ChAT from E6 onwards (Spira et al., 1987). Importantly, I could clarify that both markers are expressed in one common pool of early postmitotic cells. To further distinguish within this cell pool between future GCs, which are the first cells to differentiate (Kahn, 1973, 1974) and future ACs, I used the POU domain transcription factor Brn3a as a specific GC marker (Quina

et al., 2005; Chapters I, II). Surprisingly, the early ChAT$^+$ cells were found to express Brn3a as well. Then around E6/E7, two populations of cells emerged, out of which one set down-regulated ChAT and up-regulated Brn3a to become specified as GCs, while another set of closely abiding cells did the opposite to become specified as future SACs. To cross-check I used cellular retinoic acid binding protein (CRABP) as another early GC marker, and could establish that at E5/E6, the early ChAT$^+$ cells also co-localized CRABP in addition to Brn3a, further supporting that both the GCs and SACs shared a common pool of progenitors.

How the cells decide to become GCs or ACs within this pool of cells remains an exciting open question. As one alternative, the GCs - while transiently expressing ChAT - could induce the closely abiding cells to become SACs. Moreover, all these progenitor cells also expressed AChE, which might have direct influence on stopping proliferation (see under “fire-wall”, below), as well as differentiation and cell specification. Ultrastructural studies on cells expressing AChE in E6 chick retina cultured in vitro showed that the expression was associated to cellular differentiation (Araki et al., 1982). Increased expression of AChE by transfection in vitro resulted in advanced tissue differentiation, suggesting non-enzymatic, non-classical functions of AChE during retinal development (Robitzki et al., 1997). As mentioned above, the SACs produce ACh. While ACh is a well known excitatory neurotransmitter in the CNS and in retina, its appearance during such early developmental periods clearly suggests developmental functions other than neurotransmission, such as morphogenesis, regulation of neurite outgrowth, etc. (Layer and Willbold, 1995; Rüdiger and Bolz, 2008), as synaptogenesis itself takes place only from E12 onwards in chick retina.

The two types of starburst amacrine cells express cholinergic markers differently

From E7 onwards, I could easily distinguish two populations of ChAT$^+$ cells at the borders of the future IPL. Though the two types of SACs differentiate from a common pool of progenitors (Prada et al., 1999; this study), there exist differences in their biochemical properties, e.g., the type-II cholinergic cells displaced to the GCL express cellular retinoic acid binding protein (CRABP) transiently during their differentiation, but type-I cells do not (Stanke et al., 2008). Here, I have shown a difference in the expression pattern even between the two main cho-

linergic markers AChE and ChAT during the development of the two cell types. Until E9, the stage at which the two subbands have formed, there was no difference in ChAT expression. Interestingly, as the type-II cells began to migrate towards the GCL, they started to down-regulate ChAT in the cell soma which was reflected in the corresponding subband as well. But the cells had AChE on their surface and also along their dendrites when reaching subband d.

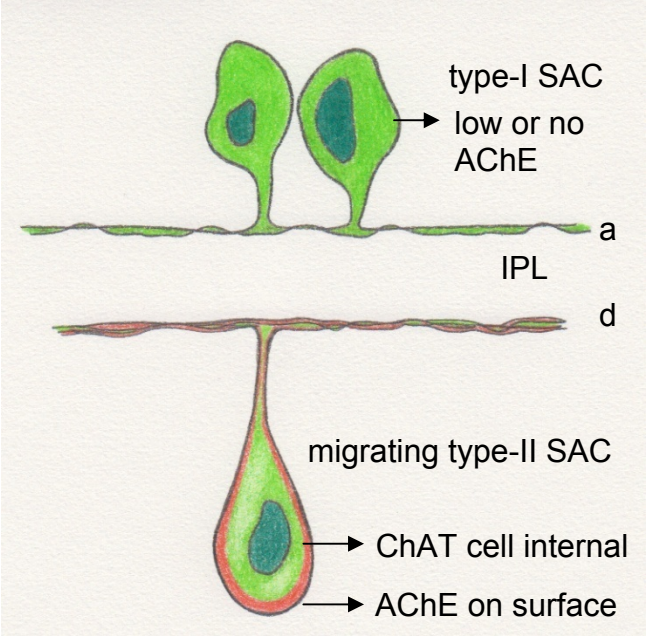

Fig. 1: Schematic diagram of type-I and type-II cholinergic amacrine cells and their expression pattern of AChE (red) and ChAT (green). ChAT, but not AChE, is strongly expressed in type-I cells. The migrating type-II cells have less ChAT, being localized cell-internally, while AChE is expressed on cell membrane. This surface-associated AChE will hydrolyze any ACh released from the cell, thus blocking any of its morphoregulatory actions (see Fig. 2). Thus, an ACh-free space can allow the cells to move further with the guidance of AChE (cf. Figs. 2, 3).

On the other hand, type-I cholinergic amacrine cells at the INL/IPL border had a stronger expression of ChAT, but weaker or no expression of AChE (chapter I). What functions could AChE perform, which is expressed on the cell surface of type-II SACs? AChE might create an ACh-free space, by hydrolyzing any ACh being released by the cells, further allowing the cells to move or migrate, as ACh itself is a strong morphogen that regulates neurite growth and cell movement. Another interpretation is that AChE might perform an adhesive function whereby these cells might be attracted towards the GCs which could express cholinesterase domain (ChED) proteins. Possible different roles of AChE have been

reviewed by Vogel-Höpker et al. (2011). A schematic representation of different roles of AChE is shown in Fig. 2 (see also, text below).

Is cholinergic network formation dependent on SACs and/or on ganglion cells?

Though it is obvious from the present study that a primary scaffold of an IPL is formed between the two SAC types, previous reports have suggested that in mammals GCs provide primary cues for IPL stratification (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1999). Other studies, however, suggested that amacrine cells might be the first to laminate the IPL, even before the ganglion cells ramify their dendrites (Bansal et al., 2000; Gunhan et al., 2002; Stacy and Wong, 2003). My findings with the bird´s retina do support the latter notion. By E8, both the type-I and type-II SACs occupied an intra-plexiform position. The cholinergic type-II cells migrated towards the GCL after E8, which results in the widening of the IPL. By that time, the two prominent cholinergic subbands a and d are already established. Thereafter, it is only the cell somas that further migrated towards the GCL, while their processes remained anchored to subband d.

Why the type-II SACs migrate into the GCL is not yet understood, however, they might do so due to some environmental cues from GCs (see below). To clarify whether GCs contribute to the migration of the type-II SACs, I analyzed by AChE and ChAT immunochemistry whether subbands a and d are being formed in a retinal explant system which does not have ganglion cells. A transgenic mouse model which lacked ganglion cells showed that the development of the IPL as well as cholinergic stratification was disturbed (Kay et al., 2004). The explant culture system used here is described in chapter III (Thangaraj et al., 2011). The GCs did not survive for more than two culture days in vitro, when the IPL and the cholinergic stratification had not yet formed. After two days in culture the type-II cholinergic cells – in absence of any GCs - migrated inwards forming a separate layer which corresponded to the location of the GCL in vivo. Both cholinergic subbands and even two non-cholinergic subbands were observed, very similar to the normal situation (chapter IV). There are no other reports on development of the IPL and cholinergic stratification in chick retina lacking ganglion cells.

Though the development of all cellular and plexiform layers was normal in explants, certain consequences of the absence of GCs cannot be ruled out. For instance, an extended migration of type-II SACs together with a down-regulation of ChAT in both cells and their processes as seen in vivo (chapter I) could not be observed in explants (chapter IV). In order to address the question of an early influence of GCs (before their loss) on SACs, I used the retinospheroid technology which is a powerful in vitro technique based upon the capacity of chicken retinal cells to reaggregate, proliferate, differentiate and re-organize into histotypic structures (Adler, 1973; Akagawa et al., 1987; further reviewed by Willbold and Layer, 1998). Thereby, the retinal tissues from E6 chick were completely dissociated to single cells. After their reaggregation, I could show that the development of an IPL could be still observed. Like in explants, GCs did not survive in retinospheres. But still SACs were able to differentiate, migrate into IPL-like areas and even begin to sub-stratify. Such a degree of stunning organizational capacities of SACs supports their leading role in IPL formation. Though these developmental capacities are interesting, the environmental cues that could drive SAC migration in absence of GCs remains to be elucidated.

Using the same retinospheroid approach, Gesine Bachmann and myself have directly tested the requirement of SACs in IPL development by performing SAC knockout studies. In that study (not part of this thesis; see thesis of Gesine Bachmann, 2012), we have knocked-out selectively and nearly completely all type-I and type-II SACs by application of the neurotoxin L-glutamate. As a result, the cholinergic IPL stratification was completely lost, documenting that SACs play a crucial role in IPL formation.

Early Müller glial cells as further candidates to support IPL stratification

Müller glial cells (MCs) are found only in retina and span its full width; they are born late during development. Though their differentiation is expected to take place after E15 in vivo, the presence of early precursors of MCs cannot be ignored. Though retinal neurons and MCs share common progenitors (Turner and Cepko, 1987), MCs show tremendous plasticity during development of the retina (Burke and Smith, 1981; Reichenbach and Robinson, 1995a; Bringmann et al., 2006). Due to their remarkable plasticity during maturation, the morphological shape, biochemical equipment and functions of MCs change continuously (Will-

bold and Layer, 1998). These early progenitor cells could be suitable candidates which might support the migration of the cholinergic cells deep into the IPL. I could show here that after application of the specific MC toxin DL-alpha aminoadipate (DL-AAA) the cholinergic stratification was completely disturbed (chapter V). But interestingly the type-II SACs still migrated into the IPL. It is to be remembered that DL-AAA shows a high affinity towards mature MCs rather than early progenitors (Olney et al., 1971; Pederson and Karlsen, 1979). This is in line with my findings that the type-II SACs might have migrated with the support of early MCs, which are resistant to the toxin but the cholinergic stratification could not be maintained anymore when the mature MCs became susceptible to the toxin.

Another unexpected result was an increase in the numbers of inner retinal cells in the retinospheroids under DL-AAA treatment. At a higher concentration of the toxin, normally the spheres do not survive in culture, demonstrating the severity of the toxin. In the present study 0.4 mM DL-AAA was used as a final concentration which proved to spare the general structure of spheres. The expression of the enzyme glutamine synthetase, which is a marker of mature MCs, was totally lost in the IPL-like areas in retinospheroids. On the other hand, the MCs were not associated with cellular apoptosis, suggesting that the present concentration of the toxin might have induced morphological changes in MCs, but not their death. Another possible explanation could be that since the toxin was present from the beginning of the culture period, it might not have allowed MCs to become completely differentiated. Under such conditions, neurons could be affected where they could shift their cell lineages either towards outer retinal cells (photoreceptors; refer to thesis of Gesine Bachmann, 2012), or towards inner retinal neurons (this study, chapter V). In mice, an intra-vitreal injection of DL-AAA caused the MCs to de-differentiate, proliferate and become photoreceptors (Takeda et al., 2008). Thus, the capacity of DL-AAA to shift retinal cell lineages needs further studies, involving gene expression studies.

Does AChE delineate and pre-structure cholinergic IPL subbands?

My studies have also revealed novel insights of how AChE expression could contribute to the very early establishment of this entire structure. At the developmental stage E6, I observed two irregular rows of $ChAT^+$ cells among the

differentiating GCs, consistent with other studies (Spira et al., 1987). Then by E7, in a cell-free space I could detect a narrow band of AChE precisely emerging in between these two rows of $ChAT^+$ cells, thereby separating the two rows into type-I and type-II SACs (chapter I). The type-I cells occupied the INL/IPL border, while the type-II cells would displace to the GCL. At E8, the narrow band of AChE had widened further, still separating the two cholinergic cell rows. It is important to notice that the IPL develops precisely in between these two cholinergic cell types, and not in between the GCs and the INL (see Fig. 3). What could such a broad and strong AChE band mean? I hypothesize that AChE lays a basis for the development of the future sublaminae. By providing an ACh-free space, this intermediate AChE band might provide guiding cues to growing neurites from ACs that progress into the cell-free IPL space (cf. Fig. 5a-h, Chapter I). By E9/E10, both type-I and type-II SACs had established their processes in such a way that the dendrites of neighboring cells joined horizontally to form continuous bands, which form the future cholinergic IPL subbands.

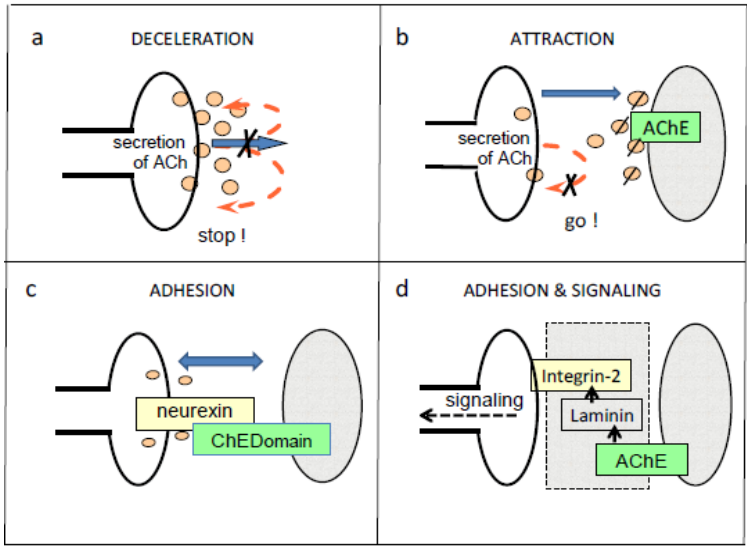

Fig. 2: Schemes of possible functioning of ACh and AChE in neurite growth, cell contact and cell signaling. (a) ACh secreted from the growth cones of cells can stop further advancement of the cell, as ACh itself is a strong morphogen. (b) When an $AChE^+$ cell is available nearby, the secreted ACh is hydrolyzed by AChE, thus neutralizing the stop signal (cf. a). In my studies, I have shown the presence of AChE on the surface of the same cell and it might perform a similar action (Fig.1, General discussion). (c) AChE can induce adhesion between two cells by interacting with cholinesterase domain (ChED) proteins, like neurexin, neuroligins etc. (d) AChE could also function in cell signaling by binding to laminin of the ECM, which in turn binds to integrin-2 to facilitate cell signaling (adopted from Vogel-Höpker et al., 2011, with permission).

Why do the processes of SACs advance towards the intermediate AChE subband and then turn horizontally? As a first possibility, AChE can exert a morphoregulatory function as an ACh-degrading enzyme (cf. Fig. 2). In fact, ACh is a well known morphogen and is believed to regulate morphogenetic cell movements, growth and differentiation of cells (Lauder and Schambra, 1999). For instance, ACh has been shown to attract or repulse axonal growth cones, depending on culture conditions (Lipton and Kater, 1989; Zheng et al., 1994; Rüdiger and Bolz, 2008).

Accordingly, any locally existing AChE will degrade all ACh released from SAC (or other) growth cones, thereby precisely defining ACh concentration gradients throughout the retinal space. Assuming that ACh is a stop signal for neurite advancement, AChE would then exert an attractive effect on SAC neurites. Then, by getting in close contact with this band, AChE could regulate further neurite growth by cell adhesive mechanisms (see above) by providing horizontal gliding rails. Thus, the growth cones of SACs would first grow towards the intermediate AChE subband and then turn horizontally (Vogel-Höpker et al., 2011). Besides many *in vitro* findings, this scheme of a directing role of AChE for IPL stratification is strongly supported by *in vivo* findings in an $AChE^{-/-}$ knockout mouse, where IPL subbands were indeed drastically disturbed (Bytyqi et al., 2004).

A *fire wall hypothesis* of AChE distribution: does AChE spatially restrict cell proliferation?

As some of my findings indicate, the distinct distributions of ChAT and AChE over the width of the entire retina may lead to a distinctively graded distribution of ACh within the retinal tissue. Since this has never been considered before, I can only speculate about its possible far-reaching significances. From E4 onwards, but most pronounced after E8/E9, nearly half of the INL was occupied by cells expressing more or less AChE (see Fig. 3). Noticeably, they formed a sharp border towards completely $AChE^{-}$ cells of the outer half retina which will comprise BCs, HCs, PRs and MCs (chapter 1); but not to forget, all cells which can still divide are restricted to this outer half retina (Willbold and Layer, 1992). Such a bulk concentration of AChE in the inner half retina will function as a sink for any ACh released from SACs; any processes originating from cells in the outer half retina would be initially attracted towards the inner retina (which is the

general direction of all neuritic growth in retina). Possibly more importantly, since all AChE$^+$ cells are postmitotic (Layer, 1983, 1990), and in the retina all of them are located in the inner half, it is most likely that AChE expression has not only a role in differentiation (e.g. neurite growth regulation), but a role in inhibiting further proliferation, e.g. effectively fixing the postmitotic state of neurons.

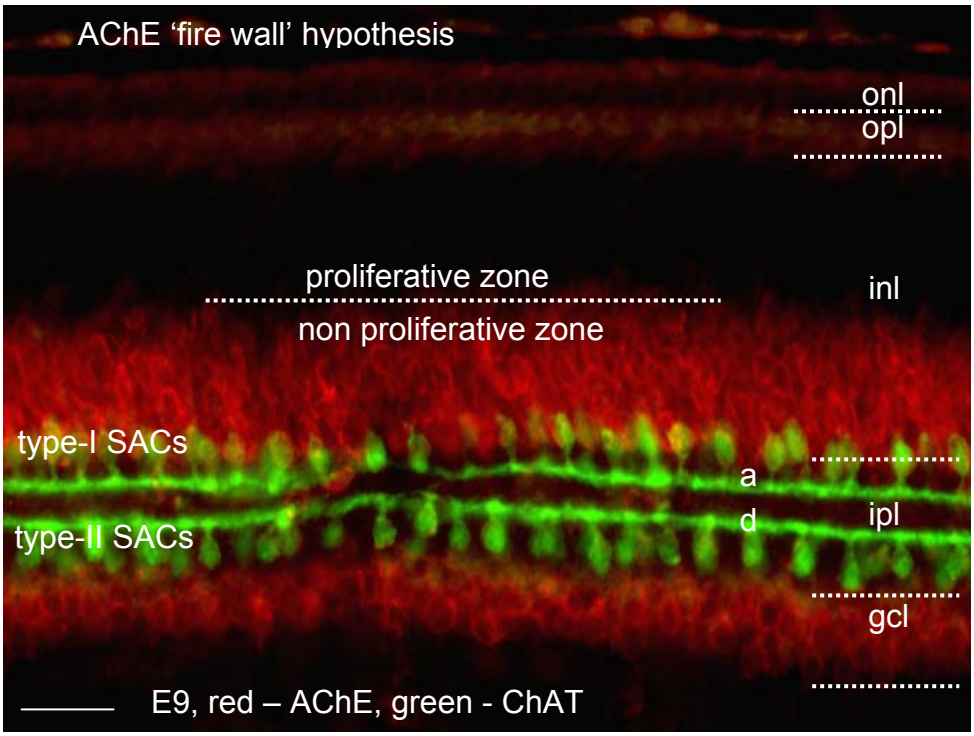

Fig. 3: Explanation of our *AChE fire wall hypothesis*. In vivo section of E9 chick retina immunostained for AChE (red) and ChAT (green). Note the two types of SACs in the ipl and their two subbands a and d. Note the distribution of AChE in inl and gcl. The inner half of the inl is filled with AChE$^+$ cells, while the outer half is completely devoid of AChE. Note that cells in the inner part of the inl do not proliferate any more, while the outer part contains cells which still proliferate. AChE itself might inhibit further proliferation, and – by degrading released ACh from SACs prevent leakage of the ACh deep into the outer retina, which otherwise may lead to excitatory (differentiative) ACh effects on cells in the inl and onl.
Abbrev: gcl – ganglion cell layer; inl – inner nuclear layer; ipl – inner plexiform layer; onl – outer nuclear layer; opl – outer plexiform layer. Scale bar – 50 µm.

Besides the above discussed cell-external, tissue-wide functions of AChE, Prof. Layer and myself hypothesize that AChE may also control ACh contents in the interior of a cell, and thereby inhibit a stimulatory effect of ACh to stimulate cell division. In this respect my finding may be significant that in type-II SACs ChAT was observed cell-internally, and AChE was expressed on the surface of the same cells. It is well established that nicotinic stimulation, and therefore ACh itself as well, is a strong proliferative stimulus (Schuller and Al-Wadei, 2010).

Ample evidence from this and other labs support this notion (Layer and Willbold, 1995; Geyer et al., 2012); for instance, in many tumours AChE is dramatically amplified (Soreq et al., 1991; Perry et al., 2002) and BChE concentrations are drastically changed. In this respect it is worthwhile mentioning that Müller cell bodies are found in the middle of the INL, e.g. exactly at the borderline of the AChE *fire wall*. MCs do not express AChE, but some BChE which often precedes the expression of AChE and has been considered a trans-mitotic marker (Layer, 1990; Willbold and Layer, 1998). In other words, a low concentration of AChE (or BChE) may convey a plastic status to cells. Interestingly, MCs have retained some plastic ability to dedifferentiate and become retinal stem cells.

These fascinating, but to some degree still speculative aspects of our *"AChE fire wall hypothesis*" were only initiated by my observations; they certainly deserve further investigations.

Concluding remarks

My work as described in this thesis has led to significant new insights into the cellular origin and development of the cholinergic system in the chick retina. Thereby, I could establish a leading role of $ChAT^+$ cholinergic amacrine cells (SACs) in IPL network formation. Furthermore, I demonstrated that IPL network formation mainly depends on SACs and on Müller cells, but not primarily on ganglion cells. The conspicuous distribution of AChE strongly suggests that the enzyme has important developmental roles during early periods of retinal development. Our *"fire wall"* hypothesis – as postulated here – suggests a prime role of AChE to define the active concentration of ACh over the entire retinal width. This in turn should contribute to both a) regulating neuronal differentiation and formation of the IPL, as well as b) restrict all proliferative activities within the inner half retina. This latter aspect needs much more attention, since it might bear significance for all nervous, and even for other non-neural tissues. In future work, the balanced expression and regulation of all cholinergic components, including the α7-nAChR, must be investigated. Further using the retina as a model system, such investigations promise to be not only much rewarding for developmental biology, but even more so for understanding the bases of many diseased states, including inflammation, cancers and dementias.

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List of abbreviations

%	Percentage
°C	degree Celsius
µ	Micro
µg	microgram
µl	microliter
µm	micrometer
Abbrev	abbreviations
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACs	amacrine cells
BCs	Bipolar cells
BChE	Butyrylcholinesterase
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	complementary DNA
ChAT	Choline acetyltransferase
cm	centimeter
CNS	central nervous system
CO_2	Carbon dioxide
CRABP	cellular retinoic acid binding protein
CS	chicken serum
DAPI	4, 6-Diamidin-2´-phenylindoldihydrochloride
DEPC	diethylpyrocarbonate
div	days in vitro
DL-AAA	DL- alpha aminoadipate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dSAC	displaced starburst amacrine cells
E	embryonic stage
et al	and others
FCS	fetal calf serum
GABA	gamma amino butyric acid

GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GCs	ganglion cells
GCL	ganglion cell layer
HBSS	Hank's balanced salt solution
HCs	horizontal cells
hr	hour
ILM	inner limiting membrane
INL	inner nuclear layer
IPL	inner plexiform layer
M	Molar
mM	millimolar
mg	milligram
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitres
mRNA	messenger RNA
ng	nanogram
OFL	optic fiber layer
OLM	outer limiting membrane
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segments
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pm	picomoles
PRs	Photoreceptors
RNA	ribonucleic acid
RNase	ribonuclease
RPE	retinal pigment epithelium
rpm	rotations per minute
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Author contributions

Chapter	**Total In %**	Planning in %			Experiment in %			Text in %		
	GT	**GT**	AG	GB	**GT**	AG	GB	**GT**	AG	GB
I	**90**	90	10	-	80	10	10	100	-	-
II	**100**	100	-	-	100	-	-	100	-	-
III	**60**	50	50	-	50	50	-	80	20	-
IV	**100**	100	-	-	100	-	-	100	-	-
V	**90**	90	-	10	80	-	20	100	-	-

GT – Gopenath Thangaraj

AG – Alexander Greif

GB – Gesine Bachmann

Chapter I
GT planned the experiment, conducted the experiment, documented the results and wrote the manuscript.

AG planned the experiment and helped in documenting the results.

GB helped in cryosectioning of chick eyes. PG supervised the work and helped in the manuscript.

Chapter II
GT planned and conducted the experiment, documented the results and wrote the manuscript.

Chapter III
GT planned and conducted the experiment, documented the results and wrote the manuscript.

AG planned and conducted the experiment, documented the results.

All figures in this chapter have been prepared by GT and AG.

Chapter IV
GT planned and conducted the experiment, documented the results and wrote the manuscript.

Chapter V
GT planned and conducted the experiment, documented the results and wrote the manuscript.

GB helped in planning and conducting the experiment and documenting the results.

Prof. Dr. Paul G. Layer supervised all my works, thereby providing significant stimuli for the design of experiments and the interpretations of my findings. Also, his expert help with editing all my manuscripts and this written thesis was substantial.

List of figures shared with other authors

Chapters I and II: all figures are mine.

Chapter III: all figures are shared between AG and me.

Chapter IV: all figures are mine.

Chapter V: Fig. 3a is adapted from GB; otherwise, all figures are mine.

Declaration

I hereby declare that the information furnished above is true up to my conscience and is reported with the consents of the corresponding co-authors.

_____
(Gopenath Thangaraj)

Consent of the co-authors

We hereby declare that we have no objection in the details furnished regarding co-authorship and the details on author contributions have been reported with our permission.

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_____
(Paul Layer)

List of Publications

Publications related to thesis

Gopenath Thangaraj, Alexander Greif and Paul G. Layer. 2011. Simple explant culture of the embryonic chicken retina with long-term preservation of photoreceptors. Exp Eye Res 93(4) 556-564.

Gopenath Thangaraj, Alexander Greif, Gesine Bachmann and Paul G. Layer. 2012. Intricate paths of cells and networks becoming “cholinergic” in the embryonic chicken retina. J Comp Neurol (In press).

Gopenath Thangaraj and Paul G. Layer. Differentiating ganglion cells transiently express choline acetyltransferase in embryonic chick retina. (Manuscript to be submitted).

Gopenath Thangaraj, Gesine Bachmann and Paul G. Layer. DL-Alpha aminoadipate induces a cell type shift towards inner retina but delays photoreceptor differentiation in chick retinospheroids (Manuscript in preparation).

Other publications

Poongothai J., Gopenath T.S. and Manonayaki S. 2009. Genetics of Human Male Infertility - Singapore Med J. (Review article) - 50(4) 336-347.

Poongothai J., Gopenath T.S. and Manonayaki S. 2008. A 386 G Transition in DAZL gene is not associated with spermatogenic failure in Tamilnadu, South India - Indian J Hum Genet 14(1) 16-19.

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Ehrenwörtliche Erklärung:

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Sämtliche aus fremden Quellen direkt oder indirekt übernommene Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und noch nicht veröffentlicht.

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